M 2014

GROWTH PERFORMANCE, ANTIOXIDANT AND INNATE IMMUNE RESPONSES IN EUROPEAN SEABASS FED PROBIOTIC SUPPLEMENTED DIET AT THREE REARING TEMPERATURES.

LUÍS FILIPE FERREIRA PEREIRA

DISSERTAÇÃO DE MESTRADO APRESENTADA

AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

DA UNIVERSIDADE DO PORTO EM

CIÊNCIAS DO MAR E RECURSOS MARINHOS

Luís Filipe Ferreira Pereira

Growth performance, antioxidant and innate immune responses in European seabass fed probiotic supplemented diet at three rearing temperatures.

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos.

Submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Doutor Rodrigo Otávio de Almeida Ozório.

Categoria – Investigador Auxiliar, Prof. Afiliado.

Afiliação – 1. Centro Interdisciplinar de Investigação Marinha e Ambiental. 2. Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Co-orientador – Professor José Fernando Magalhães Gonçalves.

Categoria – Professor auxiliar, Investigador.

Afiliação – 1. Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto. 2. Centro Interdisciplinar de Investigação Marinha e Ambiental.

“We must plant the sea and herd its animals using the sea as farmers instead of hunters. That is what civilization is all about – farming replacing hunting.”

Jacques Cousteau

Acknowledgement

Em primeiro lugar tenho de agradecer ao Dr. Rodrigo Ozório e Dr. Fernando Gonçalves por me terem aceitado como aluno para este trabalho. Em maior detalhe, o meu enorme obrigado ao Dr. Rodrigo por tudo que me ensinou ao longo deste ano, podendo honestamente afirmar que senti uma forte evolução na minha capacidade como futuro investigador ao trabalhar consigo. De igual modo com professor Fernando Gonçalves, com a sua inestimável experiência em sistemas e cultivo de peixe.

Ainda no ambiente de investigação, seria impossível não mencionar a equipa maravilha com qual trabalhei, sem mencionar nomes, obrigado a vós que vos vejo todos os dias! Um agradecimento especial aos que já voltaram a casa, obrigado Sthelio Braga e Kanokwan Sansuwan.

Agora aos que me aturam todos os dias à noite mais fins-de-semana, obrigado a equipa da Ornimundo, que muito me têm ajudado, com a companhia, amizade e compreensão.

Obrigado a todos os meus amigos, desde o meu melhor amigo, o meu irmão, á minha melhor amiga, a Carolina.

Por fim o mais importante, obrigado mãe, obrigado pai.

Abstract

Aquaculture industry growth often faces disease outbreaks wherein antibiotics are then used as control strategies. An alternative to these restricted chemicals could be the use of probiotics. Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host". The benefits of probiotic treatments are: improvements in host nutritional retention, antagonistic properties to bacterial proliferation, modulation of immune responses and anti-oxidants defenses, among others.

Temperature plays a major role in fish, since they are poikilothermic animals, i.e. internal temperature varies according to the surrounding environment. The intestinal microbiota composition in fish is, therefore, influenced by water temperature. Thus, the effectiveness of probiotic supplementation will vary with rearing temperatures. In addition, stress events may trigger a cascade of physiological changes, detectable by quantification of several analytical methods.

This study evaluated the effects of dietary probiotic supplementation on growth performance, immune and oxidative stress responses, and digestive enzyme activity in European seabass (*Dicentrarchus labrax*), reared at three different temperatures (17, 20 and 23 ºC) for 100 days. A pair-feeding method was adopted, having the daily feed being fixed to the voluntary intake of fish reared at 17 ºC, group with the lowest intake, in order to obtain similar probiotic intake among the temperature groups. A challenge stress trial (crowding combined with chasing with a pole for 15 minutes) was conducted at the end of the feeding trial.

Growth performance was not affected by the dietary treatment. Non-specific immune response (ACH50 activity) were temperature-sensitive ($P < 0.05$), with no dietary influence. Glutathione, an important antioxidant marker, was significantly affected by temperature × diet interaction. Digestive enzymes activities were significantly affected by the interaction between diet and temperature. The activity ratio of amylase/trypsin increased with temperature and dietary probiotic supplementation, an indication of metabolic flexibility of carbohydrate-protein utilization. Post-stress cumulative mortality were significantly higher in the 17 ºC control group. The current study showed that dietary probiotic supplementation conferred some protection to seabass, with temperature playing a modulatory effect.

Keywords: Probiotics; European seabass (*Dicentrarchus labrax*); Innate immune system; Oxidative stress; Digestive enzymes.

Resumo

O aquacultura frequentemente lida com surtos infecciosos, sendo os antibióticos frequentemente utilizados como estratégias de controlo. Uma alternativa a estes produtos químicos, fortemente restringidos poderá ser o uso de probióticos. Os probióticos são definidos como "microorganismos vivos que, quando consumidos em quantidades adequadas, conferem um efeito benéfico à saúde do hospedeiro". Os benefícios do uso de probióticos são: melhorias da retenção nutricional, propriedades antagonistas para com a proliferação bacteriana, a modulação da resposta imune e defesas antioxidantes, entre outros. A temperatura desempenha um papel importante nos peixes, uma vez que se tratam de organismos poiquilotérmicos, i.e. a sua temperatura corporal varia consoante a temperatura do seu ambiente circundante. A composição da microbiota intestinal do peixe é portanto, influenciada pela temperatura da água. Por isso, a eficácia da suplementação probiotica irá variar de acordo as as temperaturas de cultivo. Adicionalmente, eventos de stress podem desencadear uma cascata de alterações fisiológicas, detetáveis pela quantificação de diversos métodos analíticos.

Este estudo avaliou os efeitos da suplementação probiótica na dieta sobre o crescimento, as respostas do sistema imunológico e antioxidante e também na actividade de enzimas digestivas em robalo (*Dicentrarchus labrax*), mantidos em três regimes de temperaturas diferentes (17, 20 e 23 ºC) durante 100 dias. O método *pair-feeding* fora adoptado, tendo a taxa diária sido fixada no consumo voluntário dos peixes cultivados a 17 ºC, a fim de igualar a taxa de ingestão de probióticos entre os vários grupos. Um evento de stress (aglomeração combinada com uma perseguição de 15 minutos com um bastão) fora realizado antes da conclusão experimental.

O crescimento não fora afectado pelo probiotico. A resposta imunológica inata (capacidade hemolítica) demonstrou-se sensível à temperatura (P <0,05), mas sem a influência da dieta. A glutationa, um marcador importante na defesa antioxidante, foi significativamente afectada pela interacção temperatura × dieta. Aa actividades das enzimas digestivas foram também afectadas pela interacção entre temperatura e dieta. A proporção da actividade amílase / tripsina aumentou com a temperatura e suplementação probiótica, um indicador de maior flexibilidade metabólica na utilização de hidratos de carbono e proteína. A mortalidade pós-stress foi significativamente maior no grupo controlo a 17 ºC. Este estudo demonstrou que a suplementação probiotica conferiu alguma protecção ao robalo, tendo a temperatura sido um forte modulador.

Palavras-chave: Probioticos; Robalo (*Dicentrarchus labrax*); Sistema imune inato; *Stress* oxidativo; Enzimas digestivas.

Contents

Abstract 1

Resumo 2

Abbreviations 5

Figures list 5

Tables list 6

Charts list 6

1 Introduction 8

1.1 Aquaculture development 8

1.2 Aquaculture in Portugal 9

1.3 European seabass (*Dicentrarchus labrax*, Linnaeus, 1758) 9

1.4 Digestive system in teleost 12

1.4.1 A-Amylase 12

1.4.3 Trypsin 13

1.4.3 Chymotrypsin 14

1.4.4 Trypsin to chymotrypsin ratio 15

1.4.5 Amylase to trypsin ratio 14

1.4.6 Lipase 15

1.5 Immune system in teleost 15

1.5.1 Complement system 16

1.5.2 Peroxidase 17

1.5.3 Lysozyme 17

1.6 Oxidative stress in teleost 18

1.7 Probiotics in aquatic animals 20

1.7.1 Growth and Digestive enzymes 22

1.7.2 Immune system 22

1.7.3 Oxidative stress 23

1.8 Temperature in teleost 24

2 Objectives 25

3 Material and methods 26

3.1 Fish and facilities 26

3.2 Experimental diets 27
3.3 Experimental design 29
3.4 Experimental procedure and sampling 29
3.4.1 Samples preparation 31
3.4.2 Handling stress 33
3.5 Growth parameters 32
3.6 Humoral immune parameters 33
3.6.1 Alternative complement pathway (ACH50) 33
3.6.2 Peroxidase 33
3.6.3 Lysozyme 33
3.7 Oxidative Stress: Enzymatic and non-enzymatic analyses 33
3.7.1 Protein quantification 34
3.7.2 Lipid peroxidation 34
3.7.3 Catalase 34
3.7.4 Glutathione s-transferase 34
3.7.5 Glutathione peroxidase 34
3.7.6 Glutathione reductase 35
3.7.7 Total Glutathione 35
3.8 Digestive enzymes 35
3.8.1 Enzyme extraction 35
3.8.2 Protein quantification 35
3.8.3 A-Amylase 35
3.8.4 Trypsin 35
3.8.5 Chymotrypsin 36
3.8.6 Lipase 36
3.9 Statistics analyses 36
4 Results 37
4.1 Growth performance 38
4.2 Humoral immune parameters 40
4.3 Oxidative Stress: Enzymatic and non-enzymatic analyses 41
4.4 Digestive enzymes 43
4.5 Cumulative mortality 45

5 Discussion 46

6 Conclusion 52

7 References 53

8 Attachments 61

8.1 Laboratory analysis procedures 62

8.2 Presentations 66

Abbreviations

ROS – Reactive oxygen species.

CAT – Catalase.

GPX - Glutathione peroxidase.

GSH – Reduced glutathione.

GSSG – Oxidized glutathione.

GR – Glutathione reductase.

GST - Glutathione s-transferase.

TG - Total glutathione levels.

SOD – Superoxide dismutase.

A / T – Amylase to trypsin ratio

T / C – Trypsin to chymotrypsin ratio.

ACH50 – Alternative complement pathway.

Figures List

Fig 1: European seabass (*Dicentrarchus labrax*, Linnaeus, 1758) 9

Fig 2: Global seabass aquaculture production (tonnes). 11

Fig 3: Starch to maltose conversion trough α-amylase action 13

Fig 4: Polypeptides fragmentation by trypsin activity 13

Fig 5: Trypsin and chymotrypsin activation path. 14

Fig 6: Triacylglicerol hydrolization to glicerol and fatty acids by lipase reaction. 15

Fig 7: Complement system cascade reaction upon activation illustration. 16

Fig 8: Myeloperoxidase and eosinophilic peroxidase reaction 17

Fig 9: Reactive species of oxygen sources, defenses and consequences. 19

Fig 10: Aquatic engineering Laboratory (ICBAS – UP) 26

Fig 11: Experimental trial schematic design 30

Fig 12: Representative illustration of the stress event. 31

Tables List

Table 1: Basal diet analysis and composition (%) 28

Table 2: Total feed intake (FI, (g)) and voluntary feed intake (VFI, % BW/day) of fish feed for all the treatments. 29

Table 3: Temperature mean ± deviation trough the 70 day trial, with measurements noted 3 times a day (Celsius) 37

Table 4: Peroxidase and lysozyme activities 40

Table 5: Catalase, glutathione peroxidase and glutathione s-transferase activities 41

Charts List

Chart 1: Fish final weight mean values from all treatments 38

Chart 2: FCR mean values from all treatments 38

Chart 3: PER mean values from all treatments 39

Chart 4: DGI mean values from all treatments 39

Chart 5: Alternative complement pathway (ACH50) activity mean values from all treatments 40

Chart 6: Lipid peroxidation mean values from all treatments 41

Chart 7: Glutathione reductase activity mean values from all treatments 42

Chart 8: Total Glutathione concentration mean values from all treatments 42

Chart 9: *A*-amylase enzymatic activities mean values (U/mg Protein) from all treatments 43

Chart 10: Trypsin enzymatic activities mean values (mU/mg Protein) from all treatments. 43

Chart 11: Chymotrypsin enzymatic activities mean values (mU/mg Protein) from all treatments 44

Chart 12: Amylase / Trypsin enzymatic activities mean values (mU/mg Protein) from all treatments 44

Chart 13: Trypsin / Chymotrypsin ratio mean values from all treatments..... 45

Chart 14: Lipase enzymatic activities mean values (mU/mg Protein) from all treatments . 45

Chart 15: Cumulative mortalities results..... 46

1 Introduction

1.1 Aquaculture development

The beginning of aquaculture has multiple theories, from catch-and-hold to trap-and-crop theory, both presuming a likely rudimentary scenario, without place or time description. Such details came first by S. Y. Lin, a noted Chinese aquaculturist, setting the primordium of aquaculture in China's cultivation of common carp (*Cyprinus carpio*) around 2000-1000 B.C., dating aquaculture as far as 4000 years ago (Rabanal, 1988).

The current definition of aquaculture, according to FAO, is "the farming of aquatic organisms, including fish, mollusks, crustaceans and aquatic plants", encompassing the production of over 600 species. Finfish alone attain 66% of the production volume, of which over 80% come from inland facilities (SOFIA, 2014).

World aquaculture production continues to grow, although at a slowing rate, reports claim an all-time high in 2012 (US$144.4 billion), with China alone producing nearly half, becoming the main reason for the reduced productivity in some developed countries, e.g. the United States of America. Nevertheless, aquaculture represents the fastest growing industry in food sector, driving employment in this area to grown faster than the world's population. As cause and result, world per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012. This impressive development has been driven by a combination of population growth, rising incomes and urbanization, and facilitated by the strong expansion of fish production and more efficient distribution channels. Within the world's major producers, e.g., Russia, Iceland and Norway, global importance has only managed to stabilized at 4.32 %, after a simultaneous decrease since the ninety's.

1.2 Aquaculture in Portugal

Aquaculture in Portugal is a relatively old activity, although mostly on a familiar scale. Nevertheless, for decades that it encircles several mollusks bottom cultures (*Ruditapes decussatus*, *Crassostrea angulata* and *Crassostrea gigas*) and finfish inland cultures (*Dicentrarchus labra*x, *Sparus aurata* and *Solea vulgaris*). In the late 1960's, the first large scale production was built, located in Paredes de Coura for the production of rainbow trout (*Oncorhynchus mykiss*), the Castro & Cabero enterprise operates with intensive systems and still functional in these days.

In 2012, Portuguese aquaculture attained over 10.000 tons, a total value of 54 million euros. With brackish and marine fish representing 57.3% of total production and mollusks about 38%. Within finfish, Turbot (*Psetta maxima*) represents over 90% of fish production, followed by seabream (*Sparus aurata*) and European seabass (*Dicentrarchus* labrax).

Today there are approximately 1500 licensed aquaculture companies in Portugal, although reducing in number, these are increasing in production volume. Within these, less than 10 companies focus on breeding, most are growing companies, especially in semi-extensive and extensive conditions (INE, 2013).

1.3 European Seabass

(*Dicentrarchus labrax*, Linnaeus, 1758)

The European seabass (**Figure 1**) belongs to the Actinopterygii class (ray-finned fishes), subclass Neopterygii (new fins), Perciformes order (Perch-likes), and within this, the Moronidae family (Temperate basses), wherein we can find the Dicentrachus labrax (Resolution of ray-finnedfish phylogeny and timing of diversification).

Fig 1: European seabass (*Dicentrarchus labrax*)
Source:(Crocetta, 2014)

With an elongated body, males can reach 1 meter long, weight up to 12 Kg and live to 3 decades, with a bright silver tone trough the body. Juveniles tend to be slightly darker with black spots over the back. As fins, these have 2 sets of dorsal fins, with a total of dorsal spines of 8 - 10 and dorsal soft rays of 12 – 13. Anal fins have 3 spines and 10 – 12 anal soft rays. Posterior Operculum edge is finely serrated, with the lower edge

possessing strong forward- directed denticles. Mouth is moderately protractile and has 2 flat opercular spines and vomerine teeth are present anteriorly in a crescent band (Fishbase, 2014).

As an euryhaline (3 ‰ to full strength sea water) and eurythermic (5-28 °C) marine teleost species , seabass can be found from Eastern Atlantic to Morocco, Canary Islands, Senegal, Mediterranean and Black sea, inhabiting very different habitats, adapting to different prey, water and bottom compositions (FAOa, 2014).

Seabass are carnivores, feeding on crustaceans, mollusks and fish. Adults tend to wander often alone, predating fish, while juveniles form school groups in search for small crustaceans and mollusks. With a protractile mouth, predatory habits include suction of small preys.

Spawning occurs in batches, as a gonochoristic species, males and females gather in large groups in open sea. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations. Young larvae are then passively transported to nursery areas, as shallow water lagoons and estuaries. These remain in creeks, estuaries, backwaters, and shallow bays through their first and second years, after which they migrate to over wintering areas in deeper water, returning to larger estuaries in summer(ICES, 2013).

In seabass culture, studies have revealed temperature tolerance ranging from 2 to 32 °C (Vázquez & Muñoz-Cueto, 2014). With growth rate and feed utilization having a its best performance within the range of 22 °C to 25 °C(Person-Le Ruyet *et al.*, 2004). Temperature has shown capable to modulate immune system protein expressions (Sarropoulou *et al.*, 2010) and antibody response (Cecchini & Saroglia, 2002). In oxidative stress parameters, temperatures beyond optimal range for seabass induced lipid peroxidation and catalase activity, revealing that temperature deviations may cause responses similar to environmental contaminants (Vinagre *et al.*, 2012).

Although microflora bacteria and supplementation success thermal modulation have been described (Ibrahim *et al.*, 2004, Hagi *et al.*, 2004), more work is required to better understand how this probiotic-host relationship is affected.

European seabass production is historically known for being cultured in coastal lagoons and tidal reservoirs before the development of juveniles mass-production in the late 1960s. These were associated with the reuse of salt production evaporation ponds, where their activities took place along the year, the salt would be harvested during the high evaporation season of summer and autumn, and fish cultured during winter and

spring. But in the late 1970's France and Italy had finally attained a good management of this larvae production, making the European seabass the first non-salmonid species to be commercially cultured in Europe. Representing a very economical important fish, seabass cultures activity, quickly spread along Mediterranean countries(FAOb, 2014).

Recent reports claim a global production peak of 153 000 tonnes in 2012 (**Figure 2**). Main producers are: Greece, Turkey, Italy, Spain, Croatia and Egypt.

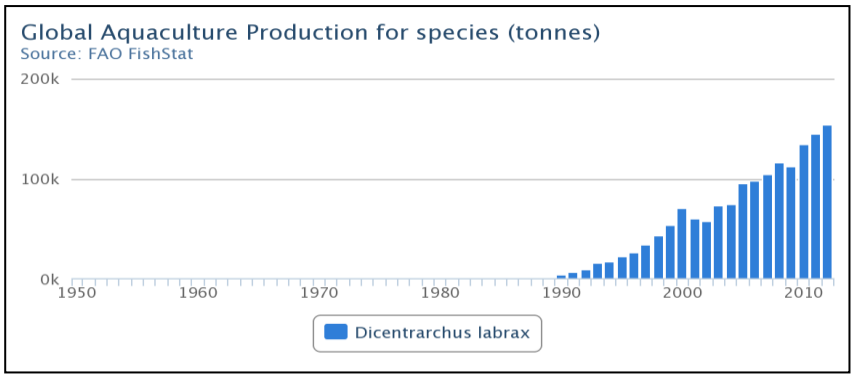

Fig 2: Global seabass aquaculture production (tonnes). Source:(FAOc, 2012).

Portugal annual seabass production peaks at 500 tonnes, a small production when facing the global 132 thousand tons produced annually, of which, Greece alone is responsible for 42 000 tonnes (FAOc, 2012).

With seabass production, scientific research around its needs increased dramatically. A natural priority was its diet. As nutritional needs, the required protein level was settled at 50% since 1988 (Hidalgo & Alliot, 1988). As protein source, seabass revealed quite dynamic as long as the absent essential amino acids were supplemented (Tibaldi & Lanari, 1991). Lipid levels revealed ideal at 12 % (Alliot *et al.*, 1974).

Starch inclusion in diet has become popular due to its use by fish metabolism as energy source, therefore sparing protein or lipids. Starch ideal levels appear to be close to 10% (Enes *et al.*, 2006a), with temperature influencing its digestibility (Enes *et al.*, 2006b).

Essential amino acids for seabass are the same as for salmonids (Kaushik, 1998), being the fish meal the best choice for protein sorce, with the only requirement for methionine – cysteine and lysine supplementation (Hidalgo *et al.*, 1987) (Tibaldi & Lanari, 1991). Mineral and vitamin supplementations appear to be reduced to vitamin C requirement, important for collagen synthesis. Since the majority of fish are not able produce ascorbic acid, this is required at a minimum of 5mg.Kg^{-1} of feed (Fournier *et al.*, 2000).

1.4 Digestive system in teleost

A better understanding of a species digestive system represents a major contribution for its rearing success. An adequate diet is closely linked to digestion and absorption; this depends on both the physical-chemical nature of the feed and from the type of enzyme activities present along the alimentary tract. Digestive enzymes are responsible for disassembling the major food components, as protein, carbohydrate and fat to their absorbable subunits. These, can then undergo transportation in the circulatory system, and be reabsorbed by the organism cells, leading to its nutrition, and therefore to growth and development. Digestion is then a progressive process, enduring from the stomach to the rectum, being expelled as feces(Smith, 1989).

Fish digestion starts in the stomach, where pepsinogen (pepsin primordium) and hydrochloric acid are produced. Pepsinogen activated by the acid, turns into pepsin and starts proteolysis, degrading peptides bonds adjacent to aromatic amino acids. Further into the digestive tract, the intestinal mucosa produces enterokinase, this, acts on the secreting pancreatic juice (trypsinogen, pancreatic amylase and lipase, and others). With the activation of trypsinogen into trypsin, a cascade of activations of other zymogens in pancreatic secretions starts, including chymotrypsinogen, proelastase, procarboxypeptidases, and some prolipases. Proteases as trypsins, chymotrypsins and caboxypeptidases A and B, will then further break down peptide chains into amino acids so these can be absorbed through the intestine brush border (Ogiwara & Takahashi, 2007, Grosell *et al.*, 2010).

Digestive enzymes activity can however be modulated by several factors, such as: diet composition (Cahu & Infante, 1994), temperature (Zhao *et al.*, 2009) and age (Kuz'mina, 1996). Therefore, evaluating digestive enzymes activities become a usefull tool in predicting the ability of a species to use different nutrient sources and/or digestibility conditions.

1.4.1 A-Amylase

The main role carbohydrates have in metabolism is of energy source upon their oxidation, acting as fuel for other metabolic functions and therefore sparing protein or lipids use as energy. Carbohydrates are used for this purpose in the form of monosaccharides, mainly glucose. Alpha-amylase is a key enzyme for carbohydrate digestion. It is secreted by the pancreas into the intestine and the pyloric caeca, where it may be adsorbed. It acts on complex polysaccharides, such as starch and glycogen, hydrolysing them up into glicose, maltose, maltotriose and a combination of branched

(1:6) oligosaccharides and glucose (Papoutsoglou & Lyndon, 2003) (**Figure 3**). Thence, studies quantify α-amylase activity for evaluation of a species capability to digest the supplemented carbohydrates. Starch supplementation represents the most common source. This can be added raw or gelatinized, studies show that partial gelatinized starch substitution over raw increases digestibility, allowing a greater reduction in protein content without mitigating growth (Peres & Oliva-Teles, 2002).

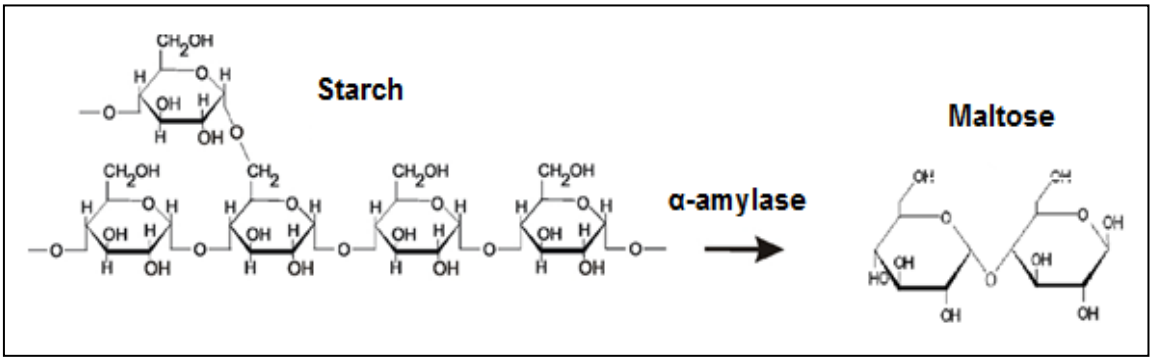

Fig 3: Starch to maltose conversion trough α-amylase action. Source: Modified from (Paul, 2012).

1.4.2 Trypsin

Trypsin [EC 3.4.21.4] appears to be the predominant protease in the midgut. Its presence has been found in pancreatic tissues from both vertebrates and invertebrates. As a serine amino acid based protease, its target for hydrolysis are peptide bonds involving the carboxyl groups of lysine and arginine residues (Smith, 1989)(**Figure 4**). Its activation comes from the action of enteropeptidase over trypsinogen. Studies claim trypsin as responsible for digesting up to 50 % of the ingested protein in carnivorous fishes (Eshel *et al.*, 1993).

Trypsin activity is usually measured using synthetic substrates. These include amide and ester derivatives of the amino acids lysine or arginine that possess only one bond that can be easily broken. Trypsin is the only pancreatic protease which can activate its own preform as well as the preforms of other proteases secreted from the pancreas, having a key position in controlling the activity of the pancreatic proteases (Hjelmeland *et al.*, 1984).

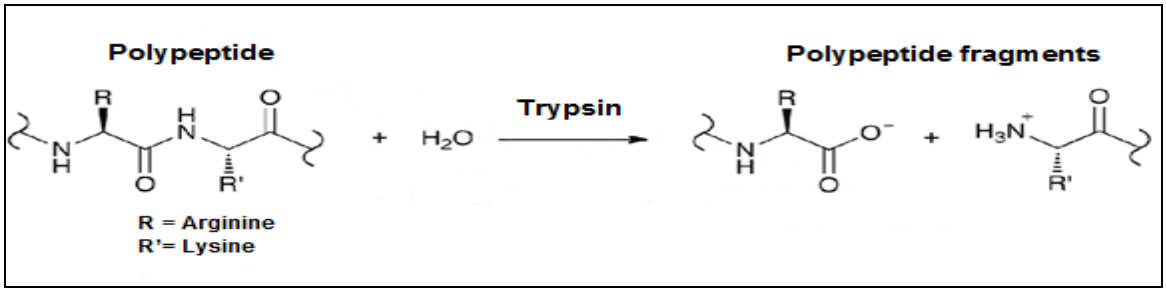

Fig 4: Polypeptides fragmentation by trypsin activity. Source: Modified from (Worthington.corp, 2014).

1.4.3 Chymotrypsin

Like trypsin, chymotrypsin is a serine type, alkaline protease produced in the pancreas and secreted in an inactive form into the lumen of the intestine. Chymotrypsin is activated upon trypsin action on Chymotrypsinogen (**Figure 5**). This enzyme then selectively hydrolyses peptide bonds on the carboxyl side of the aromatic chains of tyrosine, tryptophan, and phenylalanine and large hydrophobic residues such as methionine. Like other digestive enzymes, chymotrypsin is sensitive to temperature and pH changes (Applebaum *et al.*, 2001).

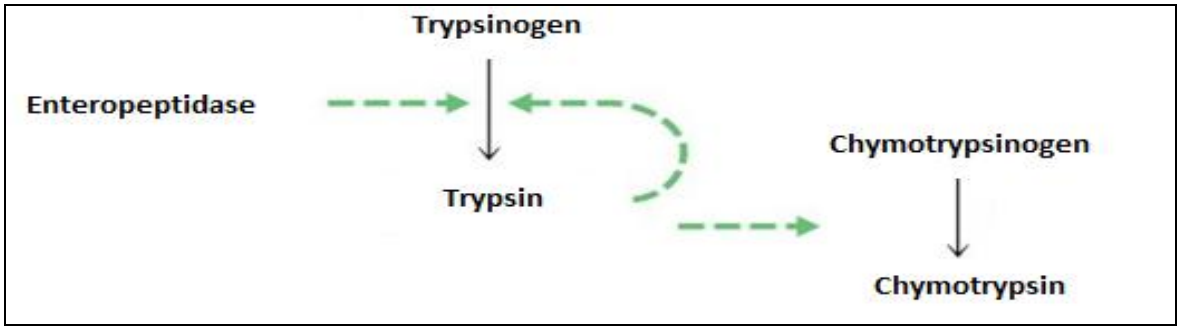

Fig 5: Trypsin and chymotrypsin activation path. Source: Modified from (Maine.University, 2014)

1.4.4 Trypsin to chymotrypsin ratio

The secretion rate of trypsin and chymotrypsin is related to feed intake and satiation levels (Rungruangsak-Torrissen *et al.*, 2006) and their activity ratio (trypsin to chymotrypsin) has been suggested as an indicator of the nutritional status of the fish, by promoting a higher absorption/transport rate of EAA for protein synthesis and plasma insulin level, hence stimulating protein synthesis(Rungruangsak Torrissen & Male, 2000).

1.4.5 Amylase to trypsin ratio

Ratio between α-amylase and trypsin is seen as an indicator of energy metabolism flexibility, showing the fish capability to use carbohydrates in substitution of proteins or lipids for energetic purposes. Often used as a sign for carnivorous feeding habits (Aragón-Axomulco *et al.*, 2012). Recent studies however tend to see this ratio from the protein saving point of view. Nonetheless, growth performance and A/T ratio, still miss correlation with better growth parameters or more efficient protein use (Thongprajukaew *et al.*, 2011).

1.4.6 Lipase

Lipids are required by four main needs in the body: energy, fatty acids, structural components and regulatory functions. If lipid recourses surpass current demand, these are stored as triglycerides within the adipose tissue beneath skin, in muscle fibers, and in the abdominal chamber. It is well known that lipids digestion differs from protein and carbohydrates digestion, since lipids are not water-soluble. To solubilize, lipids must be break down by the bile secreted from the gall-bladder. By emulsifying, lipids become vulnerable to pancreatic lipase [E.C. 3.1.1.3] hydrolization , turning triacylglicerols into diaglycerides, then monoacylglycerides and finally free fatty acids and glycerol (**Figure 6**). These are then absorved under the form of micelles, a mix of fatty acids, glycerol and bile salts(Webster & Lim, 2002).

Marine fishes require polyunsaturated n-3 fatty acids, such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) as essential fatty acids for normal growth (Cowey & Cho, 1993). The control of pancreatic secretions in fish is probably the same as in mammals, but there is no information on teleost yet. Nevertheless, studies suggest that pancreatic lipase activity results serve as a good indicator of lipase digestibility (Infante & Cahu, 1999). Studies have also suggested lipase activity modulation by dietary yeast supplementation (Tovar *et al.*, 2002a).

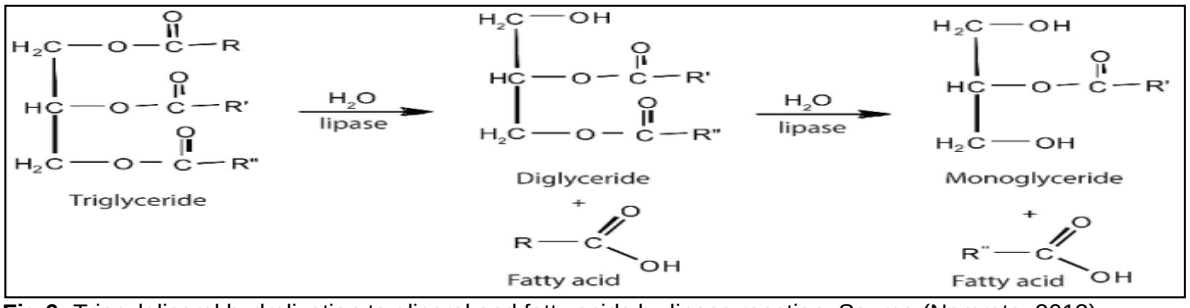

Fig 6: Triacylglicerol hydrolization to glicerol and fatty acids by lipase reaction. Source:(Namrata, 2013).

1.5 Immune system in teleost

Aquaculture rapid development has increased disease outbreaks frequency. It is well documented that the occurrence of a fish disease depends on the balance between host, pathogen and the environment, three factors with continuous interaction (Roberts, 2012).

Immune system in fish has attained interest in phylogenetic point of view, since fish were the first group of animals showing the basic aspects of the immune system of higher vertebrates such as true lymphocytes, lymphoid tissues, antibody production, T cell

cytotoxicity and long term memory (Koumans-van Diepen, 1993). As in other vertebrates, the innate immune system of fish provides the first line of immune defense. Teleost lymphoid system contains thymus, head kidney, spleen and mucosal associated to lymphoid tissue and cellular components that display humoral and cellular immune responses. Within these, non-specific cell-mediated cytotoxic, microbial killing by neutrophils and macrophages, B-cell and T-cell activities (Scapigliati *et al.*, 2002).

1.5.1 Complement system

The complement system is composed of more than 35 soluble plasma proteins and represents a very important defense system in fish, these are expressed in the liver and released into plasma. Consisting in a complex enzyme cascade and composed of several inactive glycoproteins, these can be activated by one of the three known activation routes: classical, alternative, and lectin-mediated. The functions of the complement system include lytic activity (for viruses, bacteria, and parasites) and neutralization pathogenic exotoxins (**Figure 7**). The activated proteins can also provide an alert of potential pathogens present in the host, hence contributing to the degradation of pathogens through the recruitment of immune cells and through opsonization (Boshra *et al.*, 2006).

The fish complement system have been showed to be affected by light exposure (Angeles Esteban *et al.*, 2006), environment temperature (Tort *et al.*, 1998), diet (Geay *et al.*, 2011) and more directly correlated, infection (Henry *et al.*, 2009).

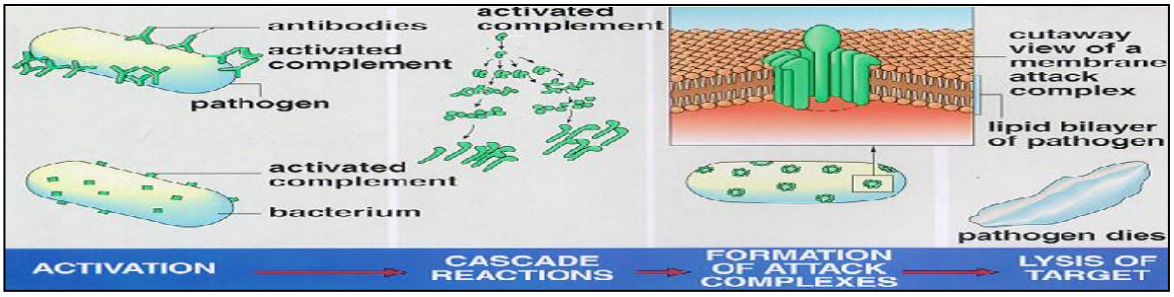

Fig 7: Complement system cascade reaction upon activation illustration. Source: (UIC, 2014)

1.5.2 Peroxidase

Both neutrophils and macrophages are important innate system components that protect the organism against microbial infection. The functions of both cells include phagocytosis, chemotaxis and bactericidal activity (Katzenback *et al.*, 2012).

Neutrophils upon activation release hydrogen peroxide and myeloperoxidase (EC 1.11.2.2), a heme-containing lysosomal glycoprotein, found predominantly in neutrophils azurophilic granules, and in much lower amounts in monocytes and some tissue

macrophages. Myeloperoxidase then catalyzes chloride ions and the hydrogen peroxide to form hypochlorous acid. Hence damaging the invading microorganisms (Klebanoff, 1968). Similar to neutrophils myeloperoxidase, eosinophills produce an eosinophillic peroxidase (EC 1.11.1.7), consisting of a heavier glycosylated chain and a lighter non-glycosylated chain. This enzyme prefers bromide over chloride as a substrate, converting it to hypobromite, toxic to infecting pathogens (Bielek, 1981) (**Figure 8**).

Peroxidases, released from the cytoplasmic granules of phagocyte participate in the oxidative responses against pathogens. Serum Peroxide levels are known to increase in response to infection (Alvarez-Pellitero, 2008). Therefore, peroxidases released into the blood are then often used as an indicator of the immunologically active status of circulating leucocytes.

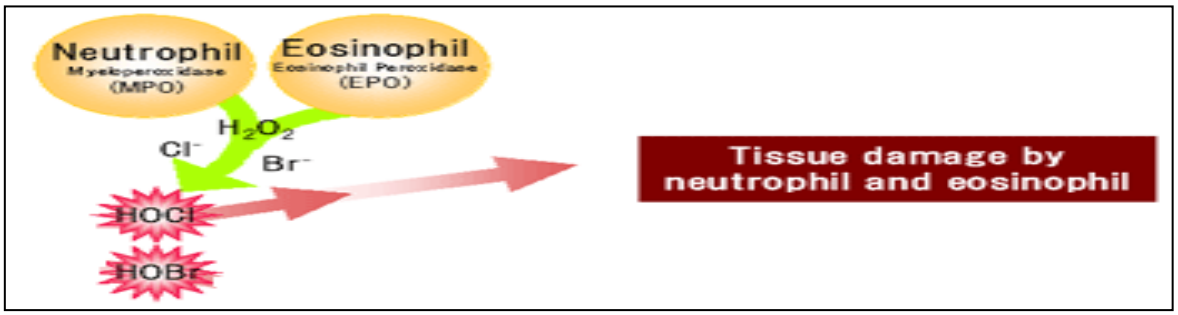

Fig 8: Myeloperoxidase and Eosinophilic peroxidase reaction. Source: Adaptation of (Jaica, 2014)

1.5.3 Lysozyme

Lysozyme was the first natural antibiotic isolated from humans in 1922 by Mr. Alexander Fleming (Nakatsuji & Gallo, 2012). As an glycoside hydrolase enzyme, lysozyme (EC 3.2.1.17) can damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Hence, lysozyme is useful tool when dealing with pathogens, especially gram positive bacteria, as these have a peptidoglycan outer layer. With of leucocyte origin, lysozyme is widely distributed in bacteriophages, microbes, invertebrates and vertebrates and its found in a large variety of animal secretions such as mucus and saliva, in many tissues including blood (Jollès & Jollès, 1984).

Studies have shown that plasmatic lysozyme activity can be modulated by several factors. Low temperatures have been shown to decrease lysozyme activity in gilthead sea bream (Tort et al., 1998), but a less linear correlation was found in Nile tilapia (*Oreochromis niloticus)*, where a slight increase revealed to increase before a plateau was reached *(Dominguez et al., 2005)* . Finally, dietary supplements provenience of probiotic

yeast (*Saccharomyces cerevisiae*) have shown capable to improve lysozyme activity as well (Torrecillas *et al.*, 2007).

1.6 Oxidative stress in teleost

A consequence of all aerobic life is the production of potentially harmful, partially reduced species of molecular oxygen radicals (ROS) which occur as a result of normal oxygen metabolism. ROS are generated by physical, chemical and metabolic processes that convert O_2 into reactive oxygen species, such as superoxide, hydroxyl radicals and non-radical hydrogen peroxide or even singlet oxygen. It has been estimated that about 1 to 3 % of O_2 consumed in animal systems is converted to ROS. Greatest incomes are the auto-oxidation of the mitochondrial electron transport chain, microsomal cytochrome P-450 and flavor-protein reductases (Livingstone, 2003). Although ROS supply the body with humoral innate protection against pathogens, as mentioned in peroxidases parameter, disturbances in the normal redox state of cells can cause toxic effect, ROS may damage all components of the cell, including proteins, lipids, and DNA. Furthermore severe oxidative stress can cause cell to trigger apoptosis, while prolonged extreme oxidative damage may cause tissue necrosis (Zong & Thompson, 2006). These effects depend upon the size of these changes. A cell can always be able to overcome small perturbations and regain its original state, without further damage. Only when the production of these aggressor outcomes the organism defense capabilities, does the animal enters the oxidative stress condition (**Figure 9**).

ROS production can be induced by external factors. Environmental pollution may well be one of the most studied sources. Transition metals (Cu, Fe) represent a major path of environmental oxidative, as they catalyze the production of hydroxyl radicals through Fenton reaction. Other chemicals like biphenyls, quinones and nitroaromatics can also induce production of superoxide by redox cycling (Sevcikova *et al.*, 2011).

Environmental influence however does not require physical or chemical hazardous conditions to induce ROS production. In aquaculture, several events common to daily maintenance may cause stress to fish, hampering fish health and growth. Studies suggest that oxidative stress in aquaculture can be correlated to several types of stressful conditions, such as: dietary influence (Olsen & Henderson, 1997), feed deprivation (Pascual *et al.*, 2003), fish crowding (Bagni *et al.*, 2007), hypoxia (Guerriero *et al.*, 2002), acute thermal conditions (Vinagre et al., 2012) and infection response (Ali *et al.*, 2011).

Within the infection response, ROS main source may well be leukocytes respiratory burst attack, as these use NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) to generate ROS, and therefore performing an aggression towards the pathogen (Alvarez-Pellitero, 2008).

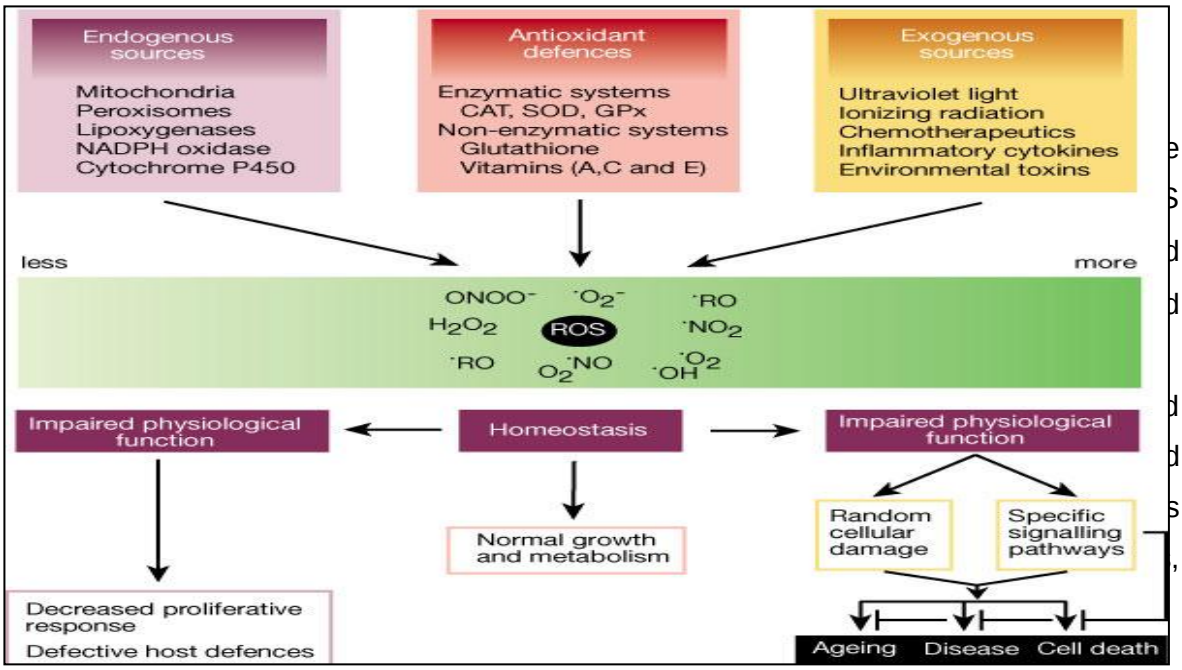

Fig 9: Reactive species of oxygen sources, defenses and consequences. Source: (Finkel & Holbrook, 2000)

Copper/zinc superoxide dismutase, catalysis the dismutation of superoxide into oxygen and hydrogen peroxide. CAT (EC 1.11.1.6.) catalysis the decomposition of hydrogen peroxides to water and oxygen, and interpreted as a protector against hydrogen peroxide. Glutathione peroxidase (GPX) (EC 1.11.1.9.), reduces lipid hydro-peroxides to their corresponding alcohols and to reduce free hydrogen peroxide to H2O, with a co-factor, glutathione (GSH). Glutathione reductase (GR) (EC 1.8.1.7), is an enzyme responsible for recovering oxidized glutathione (GSSG) to its useful state (GSH). Glutathione s-transferase (GST) (EC 2.5.1.18), another glutathione (GSH) dependent enzyme, neutralizes certain xenobiotics, inhibiting these to cause damage over the organism cells.

Finally, total glutathione levels (TG), a sum of both GSH and GSSG, a tripeptide that when in its reduced form works as an electron donor to GPX, and as cofactor for GST, and also, as a direct thiol-based antioxidant. Studies suggest that although GSH/GSSG ratio may indicate a more prompt antioxidant state, total glutathione levels drop signals a state of great use of GSH (Eroglu et al., 2014).

These molecular parameters constitute the first line of antioxidant enzymatic defense and are used as biomarkers for oxidative stress condition in a variety of marine and freshwater organisms (Eroglu et al., 2014). ROS reaction on cells lipids is considered one of the most

prevalent mechanisms of cell (Gravato & Guilhermino, 2009). Since lipids are oxidized usually through the formation of peroxides, the process of their formation has been called “lipid peroxidation”, and is quantified to determine the degree of damage that may have been caused by ROS over the lipid layers. As this damage tends to be avoided when anti-oxidant enzymes surpass the oxidants, this gives a sight of the organism vulnerability (Lushchak, 2011). As site of measurement, the liver has been the focus of toxicological studies and has indeed been shown to be very sensitive organ to oxidants presence (Ben Ameur et al., 2012). Therefore, it’s accepted that the monitoring of antioxidant enzymes activities in the liver may create a good evaluation of the antioxidant state.

1.7 Probiotics in teleost

The term, probiotic, means “for life”, its origin came from the Greek words “pro” and “bios”. Probiotics history starts with the fermentation of cheese, where milk sugars are turned to lactic acid. But the idea of the accommodation of these micro-organism within animals intestine only came in 1901, when a Russian scientist studied the human intestine microflora and suggested that ingestion of fermented milk could bring health benefits (Gismondo *et al.*, 1999).

In aquaculture intensive productions, aquatic species are subjected to high-stress conditions, increasing the probability of diseases incidence and causing a decrease in productivity. In order to control pathogenic infections, aquaculture uses chemical additives and veterinary medicines, especially antibiotics, which generate significant risks to public health by promoting the selection and persistence of bacterial-resistant strains (Martinez Cruz *et al.*, 2012). In the search for alternatives, other methodologies emerged: bacteriophages, anti-virulence therapy, chromosome II replication inhibition and the promising probiotic administration (Defoirdt *et al.*, 2011).

As the probiotic strain(s) proliferates in the intestine, this may then influence the host, acting as commensals, pathogens and even interacting within ecosystems micro-fauna. These may be administered intentionally, allowing a pursuit for an advantageous biodiversity of beneficial micro-flora for the host. With this feature, we approach the concept of probiotics, initially used to define substances excreted by an organism, capable of benefiting the growth of other, later in 1971, Sperti defines as tissue extracts capable of stimulate microbial growth, but only in 1974 Parker would define it as a microbial feed supplement (Vilà *et al.*, 2010). And so, reaching to the current definition of probiotics according to FAO, as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host", in which, for use in foods, probiotic

microorganisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut (Pineiro & Stanton, 2007).

Regarding their source, most microbial strains were initially recovered from the intestinal tract of many aquatic animals, particularly rainbow trout, Atlantic salmon and Indian carp (*Catla catla*). Currently there are about 30 recognized strains, with considering importance, the genera *Bacillus* and *Lactobacillus*. In the European Union, there is still only one probiotic based on the lactic acid bacteria strain *Pediococcus acidilactici* that has been approved as feed additive (Bactocel®I), after demonstration of beneficial effects on salmonids and shrimps(EFSA, 2012).

As administration methods, we can find: dietary supplementation, where probiotics can be directly incorporated into feed pellets at a suitable temperature; bioencapsulation, when we allow the probiotic strains to reach the intestine due to a coating, this method can use live microorganism as a delivery mechanism (e.g. artemia, rotifers); and finally, straight water enrichment, where the host get colonized trough water ingestion (De *et al.*, 2014).

The influences of probiotics already registered are diverse, encompassing improvements in nutritional retention (Mohapatra *et al.*, 2012), antagonistic properties towards bacterial proliferation (Sun *et al.*, 2010), nutrient competition towards possible pathogens (Fredrickson & Stephanopoulos, 1981), accommodation space struggle (Vine *et al.*, 2004), modulation of host's immune system responses (Taoka *et al.*, 2006). Also, recent findings indicate the properties of probiotics as promoter of better protection against oxidative stress (Chiu *et al.*, 2007, Tovar-Ramírez *et al.*, 2010).
These defensive properties, make the use of probiotics an promising alternative to antibiotics, improving the range of prophylactic treatments possible, and also aiding the on growing stringent regarding the use of chemotherapeutic products in animal in European countries (EC, 2003).

Although attractive, probiotic use, action and proliferation are affected by several factors that may limit their applications in fish models, such as water temperature, osmotic pressure and mechanical friction, pH and oxygen levels (Nayak, 2010b). Other interesting limitation, is the ‘‘quorum sensing’’ defined as a process of bacterial cell-to-cell communication. This theory defends that the homeostasis of the microflora living in their ecosystem, e.g., intestinal mucosa (Mohapatra *et al.*, 2013).

1.7.1 Growth and Digestive enzymes

Despite the promising potential benefits demonstrated in the current literature, the probiotic mechanisms which mediate host benefits are poorly understood. Digestibility however, may well be the better explained propriety. Digestive exo-enzymes have been reported in *Bacillus* genera, in fact, the bacteria isolated from the digestive tract of aquatic animals have been shown to produce chitinases, proteases, cellulases and lipases (De et al., 2014, Mohapatra et al., 2012). A better availability of exo-enzymes produced by probiotics can lead to a more efficient nutrient digestibility (Vine *et al.*, 2006). Consequently, it becomes important to quantify the activity of the digestive system enzymes in an essay with dietary probiotic supplementation.

1.7.2 Immune system

In the defense from pathogens, competitive exclusion, antagonistic compounds production and feed competition revealed success strategies. The simple idea of culturing beneficial bacterial, preventing other to house in the host intestine, therefore reducing the infection rate, represents a theory, mostly applied in fry, referred as “getting in there first” strategy (Irianto & Austin, 2002).

Probiotics can interact with the host’s immune cells such as mononuclear phagocytic cells (monocytes, macrophages), poly-morphonuclear leucocytes (neutrophils) and natural killer cells to enhance innate immune responses. Experiments report influences in the organism phagocytic activity, respiratory burst activity, lysozyme levels, peroxidases activity and complement system activity. More detailed approaches mention cytokines modulation (Nayak, 2010a). Within probiotic bacteria, *Lactobacillus* and *Enterococcus* genera appear to be the most influent in the immune system modulations. It’s most common action appears to be the improvement of complement system activity (Sun *et al.*, 2012), peroxidase(Wang *et al.*, 2008) and cytokine expression(Biswas *et al.*, 2013).

Probiotics action methods still require future work in evaluating the mechanisms by which probiotics interact with the host and pathogens. Pursuing this objective, more importance has been given to a peculiar area, the axenic and gnotobiotic organisms experiments. Axenic organisms are animals raised or treated to eliminate microbes derived from parents, gametes or environment; hence considered germ free animals. Gnotobiotic organisms are animals harboring a known microbe or microbiota, that could formerly derived from a germ free animal, posteriorly colonized by a known microbe. This area has provided interesting results, such as an adaptive trace found in zebrafish (*Danio rerio*), where its immune system has revealed tolerance to probiotic strains. More

precisely, the intestinal wall cells, enterocytes, genetic expression for alkaline phosphatases, has shown a capability to reduce the immune system response aggression upon facing microbiota lipopolysaccharide (Bates *et al.*, 2007). This divulging an evolutionary trait on hosts defensive system.

1.7.3 Oxidative stress

Probiotic supplementation has been correlated with antioxidant parameters modulation, although not completely understood, possibilities encompass two major theories: improved diet utilization, hence increasing the assimilation of dietary antioxidants from feed, and also, an active role in antioxidants activity or availability.

Studies with quantified enzymatic activities and oxidation products account greater SOD and CAT in shrimp (*Litopenaeus stylirostris*) and in leopard grouper (*Mycteroperca rosacea*) fed *Pediococcus acidilactici* and *Debaryomyces hansenii* supplemented diets, respectively (Castex *et al.*, 2009, Reyes-Becerril *et al.*, 2011). Also, a damage reduction upon lipids and DNA was observed in zebra fish fed *Lactobacillus rhamnosus* (Gioacchini *et al.*, 2014). Also, an interaction between time and dosage level of supplementation in antioxidant and oxidative damage results was found in coruh trout (*Salmo coruhensis*) fed a probiotic blend (Kefir) (Can *et al.*, 2012).

Although probiotic seem to influence the antioxidant potential, more work is necessary to understanding how is this connection being established. Nonetheless, results reveal greater resilience to ROS inducing stress events like thermal shocks, pH variations and pesticides contaminations when the organisms are fed probiotic supplemented diets (Mohapatra et al., 2013).

1.8 Temperature in teleost

As stated by Sengupta and Garrity in "*Sensing temperature*" (2013): "Temperature is an omnipresent physical variable reflecting the rotational, vibrational and translational motion of matter". For what matters in biology, temperature decides a great part of a species success and possible proliferation, and this is mostly due to temperature's capacity to mold matter. Temperature dominant modulations affect proteins and lipids re-conformation, as well as genetic modulation, playing as an on/off trigger in genetic transcription, translation or path activation (Sengupta & Garrity, 2013). A more immediate effect is the increase of kinetic energy in molecules, such as enzymes, temperature can then make molecule collisions more frequent, enabling the enzyme-subtract complex to occur more incessantly, hence increasing the reaction rate (Athel, 2004).

Expectedly temperature is often considered one of the most important variables in biochemical and physiological processes, and so, a highly concerning factor in the environment of a poikilothermic organism.

2 Objectives

The current study aimed to determine the effects of dietary probiotic supplementation on growth performances, immune system response, antioxidant parameters and digestive enzymes activity, in seabass reared at three temperatures. This experiment intends to search for the direct influence of probiotic supplementation as well as possible interactions of these influences with the auxiliary factor of temperature.

3 Materials and Methods

The current trial was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal), as according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

3.1 Fish and facilities

Fish were provided by "*L'ecloserie marine de gravelines"*, a hatchery company, situated near Calais, France. After arriving at the experimental unit, fish were kept in the quarantine system for 2 weeks at CIIMAR - UP. Then, fish were transferred to the experimental system at the aquatic production department in ICBAS – UP, and acclimatized to the rearing conditions for 15 days (**Figure 10**). During this period fish were fed a commercial diet (non-supplemented) three times a day until satiation.

Fig 10: Aquatic engineering Laboratory **(**ICBAS – UP).

The experimental system was composed of eighteen tanks of 80-liters connected to a single recirculation system (TMC® *System 5000P Marine*), at a 1.5 $L.min^{-1}$ per tank flow, with continuous aeration. Three water temperatures were tested (17, 20 and 23 ^{o}C) in a 2 x 3 factorial design. Six 17ºC tanks were acclimatized by pre-chilling the water through two aquaria chiller (Teco® – Model TR60), the 23 ºC tanks each with 2 thermostats heaters (Trixie® – 200w), and the 20 ºC tanks were obtained by acclimatization of room atmosphere trough air conditioning set to 20 ºC and also by the total water mixing (17 ºC and 23 ºC) in the water reservoir, allowing the 20 ºC mid-term.

3.2 Experimental diets

The basal diet (**Table 1**) was prepared without probiotic supplementation (control). Half of the basal diet was used as control and the other half was supplemented with probiotic blend (*Bacillus sp*., *Pediococcus sp*., *Enterococcus sp*., and *Lactobacillus sp*.) at 3g per kilogram feed (3 x 10^9 CFU/Kg feed).

All diets were formulated and manufactured by Sparos.Ltd, the main ingredients were grinded (below 250 micron) in a micro-pulverizer hammer mill (Hosokawa Micron, SH1, the Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain). After the incubation the probiotic mixtures were added to the feed mash under continuous mixing at 3g per feed kilogram ratio. All diets were manufactured by temperature controlled-extrusion (30-35 ºC) with pellet size of about 1.5 to 2.0 mm by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were left overnight to dry at 45^{o}C. For preservation, diets were stored at 4 ^{o}C, throughout the duration of the feeding trial.

Table 1: Basal diet analysis and composition (%). Experimental diet differed only in the probiotic supplementation with a probiotic blend, composed by 4 bacteria strains (*Bacillus spp*., *Pediococcus spp*., *Enterococcus spp*., *e Lactobacillus spp*.).

Control Diet composition	%
Fishmeal 70 LT (Norvik)	32.00
Fishmeal SOLOR	15.00
CPSP G	5.00
Squid meal	2.50
Wheat gluten	3.00
Corn gluten	4.00
Soybean meal 48	6.00
Rapeseed meal	5.00
Wheat meal	7.40
Peas gelatinized (Aquatex 8071)	8.00
Fish oil	9.30
Vit & Min Premix PV01	1.00
Choline chloride	0.10
Soy lecithin	0.50
Binder (guar gum)	1.00
Antioxidant liquid (Naturox)	0.20
Content	%
Crude protein	50.03
Crude fat	16.69
Fiber	1.20
Starch	9.63
Gross Energy	20.47
Lys	3.54
Met + Cys	1.95
Total P	1.22
Available P	1.05

3.3 Experimental design

The experimental trials were conducted using seabass in a fast growing stage, reared under three water temperature conditions (17, 20 and 23^{o} C) in a 2 x 3 factorial design. Fish were fed either a control or a probiotic diet for 100 days, 3 times a day by means of automatic feeders (8:00-13:00-18:00). Diets were fed at a fixed feeding level. The trial were carried out at pair feeding, i.e. at the end of the trial, all aquaria received approximately the same amount of feed (**Table 2**), fixed according to the voluntary feed intake of the fish groups reared at the lower temperature (17 ºC).

Table 2: Total feed intake (FI, (g)) and voluntary feed intake (VFI, % BW/day) of fish feed for all the treatments.

	Control			Probiotic		
Temperature	**17 ºC**	**20 ºC**	**23 ºC**	**17 ºC**	**20 ºC**	**23 ºC**
FI (g)	**30.7±0.5**	**36.8±0.4**	**40.9±0.5**	**30.7±0.2**	**35.8±0.4**	**39.4±0.7**
VFI (% BW/day)	**1.83±0.04**	**1.81±0.03**	**1.73±0.05**	**1.71±0.02**	**1.68±0.03**	**1.74±0.02**

3.4 Experimental procedure and sampling

After fish arrival, these were equally distributed into 18 tanks at a density of 26 fish (initial body weight: 12.8 ± 0.5 g) per tank, (4 Kg.m^{-3}). After acclimatization that lasted for 2 weeks, triplicate tanks were conditioned to one of the three testing temperatures (17, 20 or 23 ºC). All tanks were kept in the same recirculation systems with continuous aeration with seawater (**Figure 11**), providing a flow of approximately 1.5 L water/ min per tank. Oxygen levels near saturation (9.7 mg.L^{-1}), pH (8 to 8.5), ammonia (< 1mg / L) and nitrite (< 2 mg / L) were daily monitored. Temperatire was analysed at every tank 3 times a day (**Table 3**). Photoperiod was set for 12:12 light:dark, and water sterilization was provided by 8 ultraviolet lamps of 54 watts each.

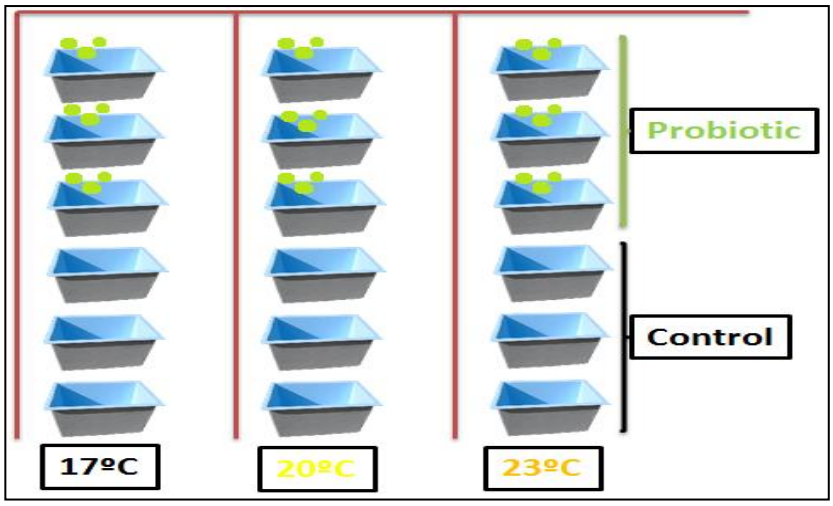

Fig 11: Experimental trial schematic design. Six tanks at each temperature (17, 20 and 23 ^{o}C). With three tanks of each temperature for each diet (Control and experimental), in a total of 18 tanks of 80 liters each.

3.4.1 Samples preparation

Two samplings were performed, at day 70 and 100. At day 70, four fish were removed from each tank and immersed in crushed ice for hypothermic anesthesia. These were then weighted and sampled for blood before cervical dislocation, followed by liver extraction. The blood was immediately centrifuged for plasma sampling, later to be used for the immunological parameters, while the liver was stored at -80 ºC for posterior homogenization. This sampling would then contribute for growth performance, immune system responses and antioxidant parameters quantification.

At the hundredth day, an acute stress would be conducted to all tanks previously to sampling. After this, 4 fish were removed per tank. These were then immersed in crushed ice for hypothermic anesthesia and then executed by cervical dislocation. Intestine would then be sampled from all 4 fish.tank^{-1}, for posterior digestive enzyme activity quantification. Finally, a total of 18 fish would remain in each tank for accumulative mortality quantification.

3.4.2 Handling Stress

To simulate a handling stress, capable of replicating a common acute stress in aquaculture production, we followed a procedure carried out (Olsen et al., 2005). This was characterized by an initial crowding, increasing animal density by 6 fold (from 4.3kg m^{-3} to 25.5kg m^{-3}), followed by a 15 min chase challenge with a pole (**Figure 12**). No other alterations were made in water flow, rearing temperature or oxygenation conditions during this event. Mortalities were registered for 10 days to perform an accumulative chart.

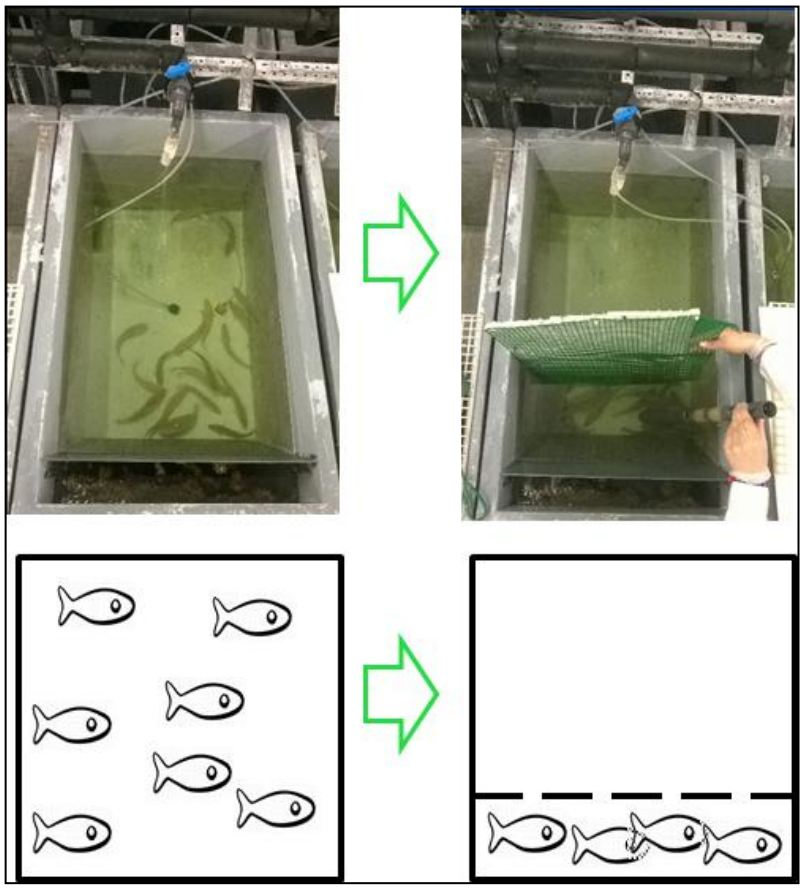

Fig 12: Representative illustration of the stress event. Composed of a crowding that increased fish density 6 times fold. Followed by a pole pursuit for 15 minutes.

3.5 Growth parameters

All fish were weighted at the beginning and end of trial. From these measurements the following parameters were calculated, feed conversion ratio (FCR), daily growth index (DGI) and protein efficiency rate (PER).

$$FCR = \frac{Feed\ consumption}{Body\ weight\ gain}$$

$$PER = \frac{Wet\ weight\ gain}{protein\ intake}$$

$$DGI = \frac{[100\ x\ (fish\ final\ weight\ 1/3 - fish\ initial\ weight\ 1/3)]}{days}$$

3.6 Humoral immune parameters

3.6.1 Alternative complement pathway (ACH50)

The ACH50 was determined according to Sunyer et al., (1995). The main principle of this procedure is to evaluate the required plasma sample to have 50% hemolysis in the added rabbit red blood cells according to the Sunyer method (Sunyer *et al.*, 1995). The alternative complement pathway (ACH50) units were defined as the concentration of serum giving 50% hemolysis of rabbit's blood cells. All analysis were conducted in triplicates.

3.6.2 Peroxidase

The total peroxidase content present in the plasma was measured according to Quade and Roth (Quade & Roth, 1997). Using TMB (3.3′.5.5′-tetramethylbenzidine) as substrate, in the presence of hydrogen peroxide. Final unit is presented as enzymatic units, with one enzymatic unit (EU) being defined as the amount producing an absorbance change of 1.

3.6.3 Lysozyme

Lysozyme concentration was determined by the turbidimetric assay as described by Ellis method (Ellis, 1990). By measuring its action upon *Micrococus lysodeikticusa* bacteria. Final unit is presented as enzymatic units made by the convention of 1 EU = 0.001 absorbance units.minute^{-1}.

3.7 Oxidative Stress: Enzymatic and non-enzymatic analyses

After liver extraction, a liver homogenate was made with a K-phosphate buffer (pH 7.4, 0.1M) in a 1:15 (p / v) ratio. For the lipid peroxidation analysis, 150 µl of liver solution were add with 2.5 µL BHT (2.6-Di-tert-butyl-4-methylphenol) at 4% in methanol, for preservation, and stored at -80 ºC until quantification. The remaining enzymes (CAT, GPX, GR and TG) samples were prepared by centrifuging liver homogenate at 10000 g forces for 20 min at 4 ºC, here post-mitochondrial supernatant (PMS) was isolated and stored in separate microtubes at -80 ºC. For CAT and GST quantification, the soluble protein content required to be close to 0.7mg.ml^{-1} concentration. For this calibration a previous protein quantification and adequate dilution were made.

3.7.1 Protein quantification

Protein quantifications were required for protein levels calibration (CAT and GST) and final activity unit reference in all anti-oxidant enzymes. This quantification followed the Comassie binding principle of Bradford (Bradford, 1976). Final unit is protein mg. ml^{-1}.

3.7.2 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS essay using thiobarbituric acid as a reagent (Ohkawa *et al.*, 1979). TBARS essay then measures malondialdehyde (MDA) present in the sample, and represents the main product of lipid peroxidation. Final unit presents as nmoles of MDA formed.mg protein^{-1}.

3.7.3 Catalase

With the soluble protein levels near 0.7 mg.ml^{-1}, activity quantification followed the principle of catalase action on peroxide hydrogen as described by Clairborne (Claiborne, 1985). Final unit is presented as hydrogen peroxide µmol.min^{-1}.protein mg^{-1}.

3.7.4 Glutathione s-transferase

With the soluble protein levels near 0.7 mg.ml^{-1}, the analysis was as described by Habig (Habig *et al.*, 1974). Where CDNB (1-chloro-2,4-dinitrobenzene) is conjugated with GSH, forming the measurable conjugate of GSH-CDNB (1-chloro-2, 4-dinitrobenzene). Final unit is expressed in pmol GSH-CDNB conjugate formed.min^{-1}.protein mg^{-1}.

3.7.5 Glutathione peroxidase

Glutathione peroxidase quantification followed the method of Mohandas, (Mohandas *et al.*, 1984). In this method the reaction is measured by the formation of oxidized NADPH (NADP+), and presented in the final unit of NADP+ pmol.min^{-1}.protein mg^{-1}.

3.7.6 Glutathione reductase

The measure of glutathione reductase in an assay based on reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to reduced glutathione (GSH), generated from an excess of oxidized glutathione (GSSG) as described by Cribb (Cribb *et al.*, 1989). Again, the activity is quantified by the formation of oxidized NADPH (NADP+), and presented in the final unit of NADP+ nmol.min^{-1}.protein mg^{-1}.

3.7.6 Total Glutathione

Total glutathione is quantified by reaction of GSH with DTNB (5.5'-dithio-*bis*-2-nitrobenzoic acid), which produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) quantified at 412 nm, as described by Baker (Baker *et al.*, 1990). During the reaction the GSSG is concomitantly reduced to GSH, hence enabling the measure of all glutathione. Final unit is presented as TNB µmol.protein mg^{-1}.

3.8 Digestive enzymes

3.8.1 Enzyme extraction and protein quantification

The extraction of the digestive enzymes was made through the homogenization of the intestine as described by Torrisen 2007 (Rungruangsak-Torrissen, 2007). Protein quantification was conducted according to Lowry (Lowry *et al.*, 1951), following the folin phenol method. Final unit expressed as protein mg. ml^{-1}.

3.8.2 A-Amylase

Amylase activity was measured from the increase in reducing sugar (maltose) by the hydrolysis of α-D (1,4) glycosidic bond in polysaccharides, and stained with 3.5-dinitrosalicylic acid (DNS) as described by Bernfeld (Bernfeld, 1951). Final unit is expressed as enzymatic unit per protein mg.

3.8.3 Trypsin

Trypsin activity was determined by the Torrisen 1984 (Torrissen, 1984), using benzoyl-L-arginine-*p*-nitroanilide as substrate. Final product (nitroaniline) was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic milli enzymatic units per protein mg.

3.8.4 Chymotrypsin

Chymotrypsin activity was determined by incubating Torrisen and Sundby 2000 method (Rungruangsak-Torrissen & Sundby, 2000), using succinyl-Ala-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. Similar to trypsin essay, nitroaniline was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic milli enzymatic units per protein mg.

3.8.5 Lipase

Lipase activity assay was performed using ρ- nitrophenyl substrate as described by Winkler and Stuckmann (Winkler & Stuckmann, 1979). The formation of p-nitrophenol is then quantified by reading at 410 nm. Final unit is expressed as the other enzymatic activities, as enzymatic milli enzymatic units per protein mg

3.9 Statistics analyses

One-way and two-way ANOVA analyses were carried out using R Studio Desktop v0.98.1028 - Windows XP/Vista/7/8 software package. Data transformation was applied when homogeneity and normality of the samples were not achieved. Tukey test was used for pairwise comparisons between treatments. Confidence level of 95% was considered in all statistical analysis.

4 Results

No mortalities were registered during the 100 days of experiment. Temperatures were measured daily to ensure the experimental design (**Table 3**).

Table 3: Temperature mean ± deviation through the 100 day trial, with measurements noted 3 times a day (Celsius).

Treatment	Experiment unit	ºC
17ºC Control	**Tank 1**	17.0±0.7
	Tank 2	17.1±0.8
	Tank 3	17.1±0.8
17ºC Probiotic	**Tank 1**	17.0±0.8
	Tank 2	17.1±0.8
	Tank 3	17.0±0.9
20ºC Control	**Tank 1**	20.2±0.6
	Tank 2	20.2±0.6
	Tank 3	20.3±0.4
20ºC Probiotic	**Tank 1**	20.3±0.4
	Tank 2	20.1±0.4
	Tank 3	20.1±0.5
23ºC Control	**Tank 1**	23.2±0.3
	Tank 2	23.2±0.3
	Tank 3	23.2±0.4
23ºC Probiotic	**Tank 1**	23.2±0.4
	Tank 2	23.1±0.4
	Tank 3	23.2±0.3

4.1 Growth performance

Fish final weight

Fish grew from the initial mean of 12.8 ± 0.5 g to a final 32.1 ± 6 g in the 100 day trial (**Chart 1**). Weight gain were significantly different between fish reared at 17 ºC treatments and 20ºC ($P < 0.01$), between 20 ºC and 23 ºC ($P=0.01$) and between 17 ºC to 23 ºC ($P < 0.01$). No significant differences were found between probiotic supplementation treatments.

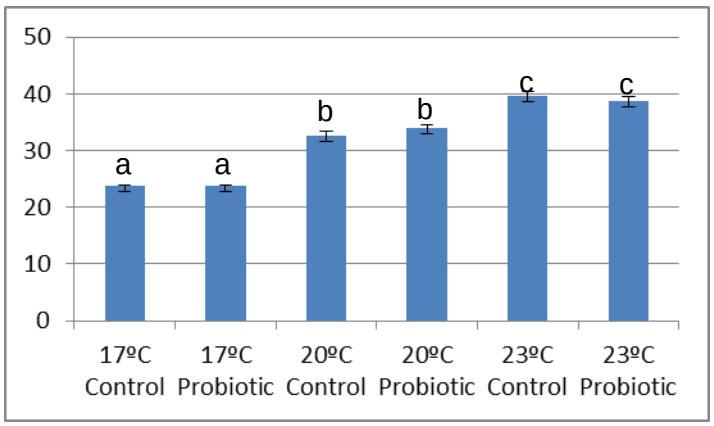

Chart 1: Fish final weight mean values from all treatments (g). (ANOVA, *P*<0.05)

Feed conversion ratio – FCR

Feed conversion ratio (FCR; **Chart 2**) were significantly different between temperatures, having the lowest temperature (17 ºC) an average FCR of 2.8, significantly greater than the 20 ºC trials (*P*-value < 0.01) and nearly twice higher than fish reared at 23 ºC (*P*-value < 0.01).

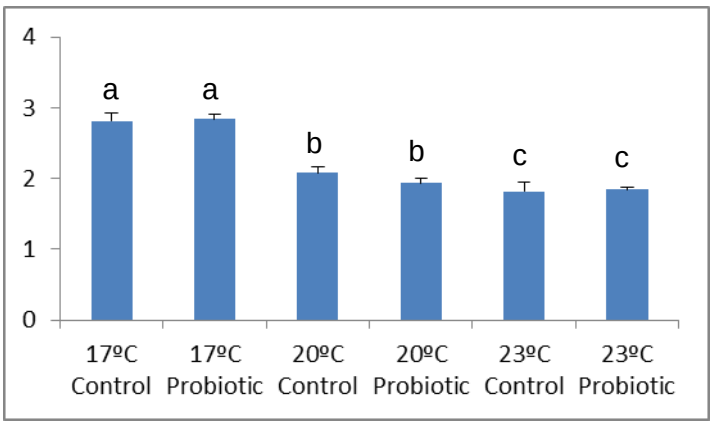

Chart 2: FCR mean values from all treatments. (ANOVA, *P*<0.05).

Protein efficiency rate – PER

Protein efficiency rate (*PER;* **Chart 3**) followed the same pattern as *FCR* (**Chart 2**), with significant differences between all temperatures (*P*-value < 0.01). But again, no significant interaction was found in a Two-way ANOVA.

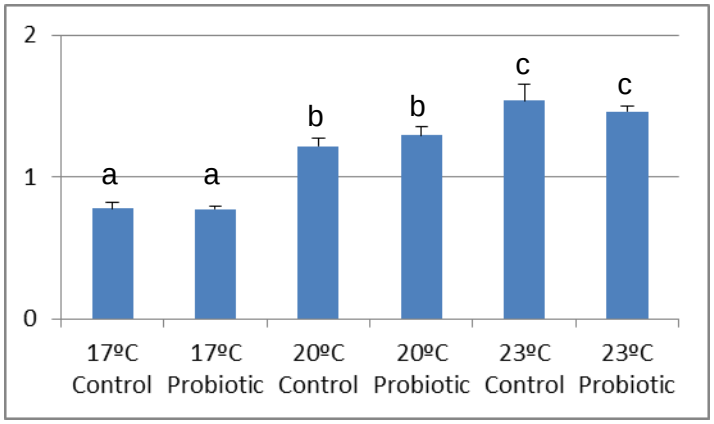

Chart 3: PER mean values from all treatments. (ANOVA, *P*<0.05).

Daily growth index- DGI

Growth rate (DGI; **Chart 4**) increased with temperature upper progression, showing significant difference between all temperatures (*P*-value < 0.01). Two-way ANOVA showed no significant difference between treatments.

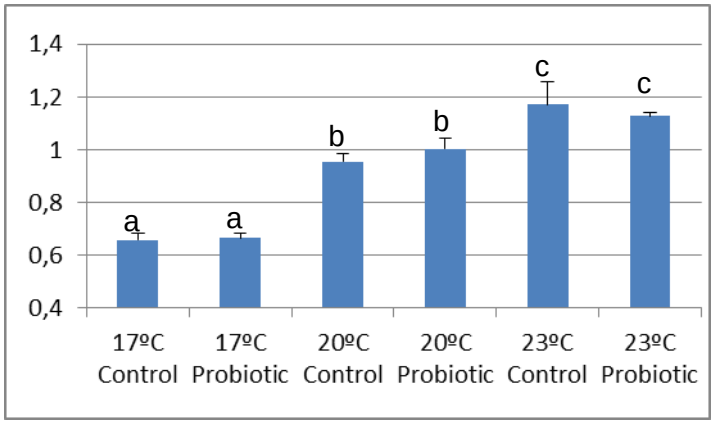

Chart 4: DGI mean values from all treatments. (Unit = % BW.day^{-1}); (ANOVA, *P*<0.05).

4.2 Humoral immune parameters

Alternative complement pathway (ACH50)

ACH50 activity (**Chart 5**) revealed differences between temperatures, with 17 ºC group having higher values than the 20 ºC and 23 ºC groups (*P*-value < 0.01).

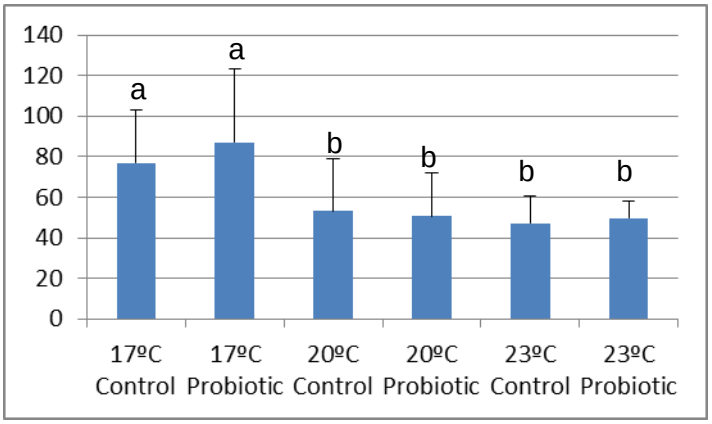

Chart 5: Complement system activity mean values from all treatments. (Unit= ACH50 Unit.ml^{-1}); (ANOVA, *P*<0.05).

Peroxidase and lysozyme activities

No significant differences were found in peroxidase and lysozyme activities (**Table 4**), although both activities revealed a low result in 1 treatment (23 ºC probiotic and 17 ºC control respectively). The characteristic high deviations in these immune system parameters quantifications may have prejudice the statistical analysis (ANOVA, *P*<0.05).

Table 4. Peroxidase (EU) and lysozyme (EU.min^{-1}.ml^{-1}) activities (mean ± SD, n=4 fish.treatment^{-1}).

	Control			Probiotic		
	17 ºC	20 ºC	23 ºC	17 ºC	20 ºC	23 ºC
Peroxidase	**24.3±9.06**	**32.7±21.7**	**26.7±13.7**	**34.7±19.9**	**40.2±23.3**	**12.5±4.1**
Lysozyme	**275.9±93.6**	**384.3±115.1**	**404.6±176.2**	**333.3±119.2**	**395.4±140.4**	**379.6±45.2**

4.3 **Oxidative Stress:** Enzymatic and non-enzymatic analyses

Catalase, glutathione peroxidase and glutathione s-transferase activities

All the anti-oxidant enzymatic activities (**Table 5**) measured did not show any significant differences between treatments. High deviations within fish of the same experimental conditions may have eluded possible distinctions; (mean ± SD, n=12 fish.treatment^{-1}).

Table 5. Catalase (umol.min^{-1}.mg Prot^{-1}), glutathione peroxidase (pmol.min^{-1}.mg Prot^{-1}) and glutathione s-transferase activities; (pmol.min^{-1}.mg Prot^{-1}); (mean ± SD, n=12 fish.treatment^{-1}); (ANOVA, *P*<0.05).

	Control			Probiotic		
	17 ºC	20 ºC	23 ºC	17 ºC	20 ºC	23 ºC
Catalase	**1.5±0.7**	**1.1±0.4**	**1.0±0.3**	**1.5±0.7**	**0.8±0.4**	**1.5±0.9**
Glutathione peroxidase	**2.1±1.2**	**4.0±1.4**	**3±3**	**4.2±3.0**	**2.2±0.79**	**2.1±0.54**
Glutathione s-transferase	**63.2±52.4**	**65.3±36.6**	**67.2±42.0**	**84.1±80.2**	**34.6±21.8**	**109.3±71.4**

Lipid peroxidation

LPO (**Chart 6**), as a form of damage from ROS over the cells lipid layers, appeared to show a clear tendency for greater damage at the lowest temperatures, with significant differences between all temperatures groups (*P*-value < 0.01); (mean ± SD, n=12 fish.treatment^{-1}).

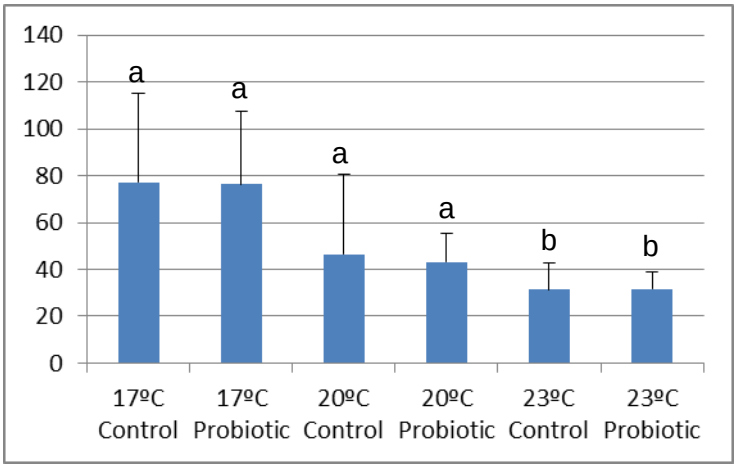

Chart 6: Lipid peroxidation mean values from all treatments. (Unit = nmol TBA.g^{-1}); (ANOVA, *P*<0.05).

Glutathione reductase

In glutathione reductase (**Chart 7**) results, mid temperature (20 ºC) treatments demonstrated significant differences within diets (*P*-value= 0.04); (mean ± SD, n=12 fish.treatment^{-1}).

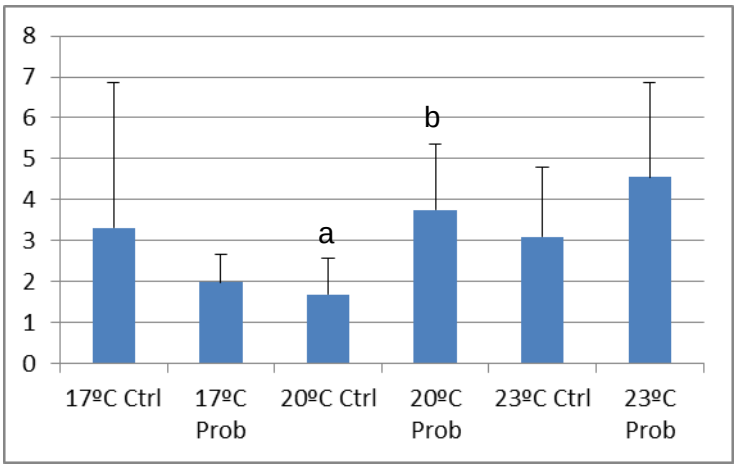

Chart 7: Glutathione reductase activity mean values from all treatments. (Unit = nmol.min^{-1}.mg Prot^{-1}); (ANOVA, *P*<0.05).

Total Glutathione

Total Glutathione concentrations (**Chart 8**) revealed significant differences between temperatures and diets. Having the 17 ºC and 20 ºC trials exhibiting an increasing effect in the supplemented diets treatments (*P*-value= 0.03).This not confirmed in the 23 ºC trials. Demonstrating an interaction between our two independent factors, temperature and diet, where the 23 ºC appear to shift the tendency for the probiotic to increase TG values (*P*-value < 0.01); (mean ± SD, n=12 fish.treatment^{-1}).

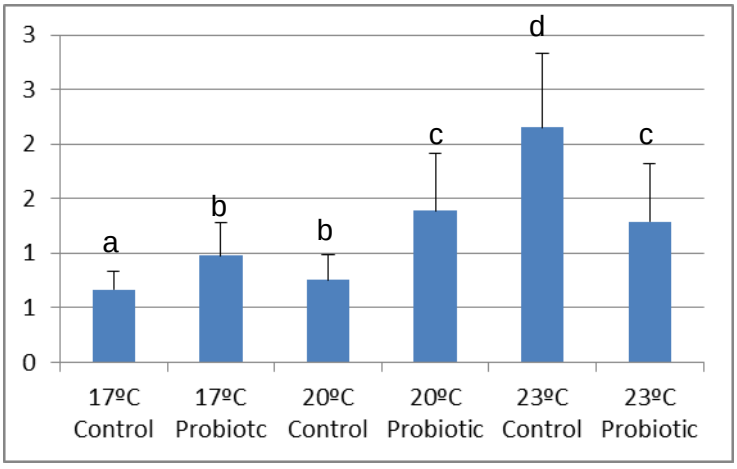

Chart 8: Total Glutathione concentration mean values from all treatments. (Unit = (umol.g^{-1}); (ANOVA, *P*<0.05).

4.4 Digestive enzymes

A-Amylase

Alpha-amylase activities (Chart 9) showed a significant difference between the 23 ºC rearing treatments and the remaining 17 ºC and 20 ºC treatments (P-value= 0.04 and 0.03 respectively); (mean ± SD, n=12 fish.treatment-1).

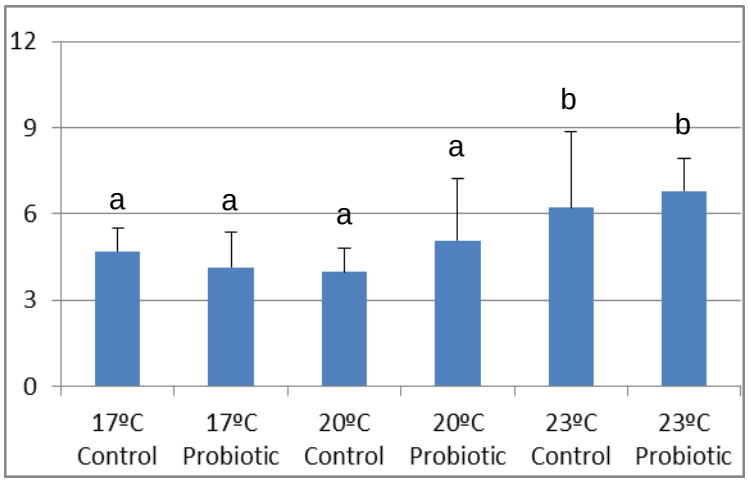

Chart 9: A-amylase enzymatic activities mean values from all treatments. (Unit = EU.mg Protein^{-1}); (ANOVA, *P*<0.05).

Trypsin

Trypsin enzymatic activities (**Chart 10**) showed a significant difference between temperatures, being the 17 ºC treatments results significantly higher than the 20 ºC and 23 ºC groups (*P*-value < 0.01); (mean ± SD, n=12 fish.treatment^{-1}).

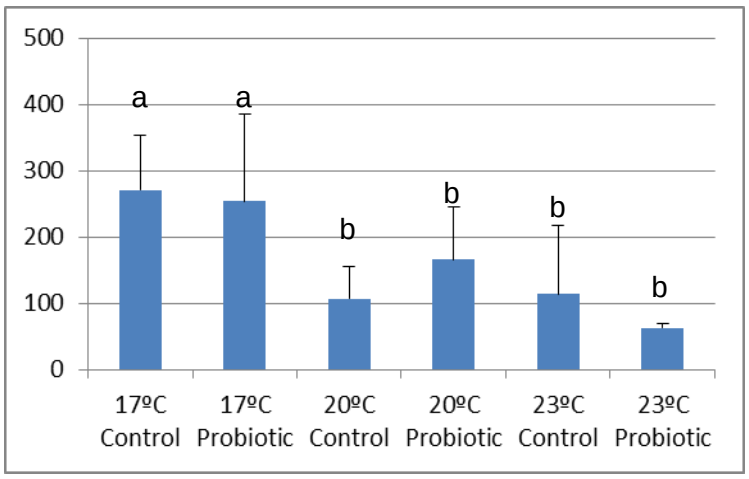

Chart 10: Trypsin enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein^{-1}); (ANOVA, *P*<0.05).

Chymotrypsin

Chymotrypsin enzymatic activities (**Chart 11**) showed a significant difference between temperatures, having the 17 ºC treatments values as significantly higher than the 20 ºC and 23 ºC groups (*P*-value < 0.01). A two-way ANOVA showed a temperature-diet interaction, since the increasing tendency of the probiotic diet on the chymotrypsin activity values, changes in the lowest rearing temperature, 17 ºC (*P*-value < 0.01); (mean ± SD, n=12 fish.treatment^{-1}).

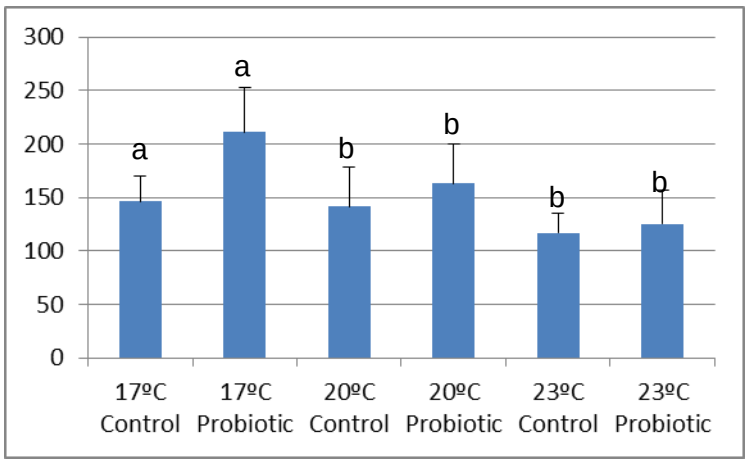

Chart 11: Chymotrypsin enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein^{-1}); (ANOVA, *P*<0.05).

Amylase / Trypsin ratio

Amylase / Trypsin ratio (**Chart 12**) showed a significant difference between all temperatures, being the 23 ºC treatments results significantly higher than the 20 ºC and 17 ºC (P-value= 0.01). No difference was found between diets; (mean ± SD, n=12 fish.treatment-1).

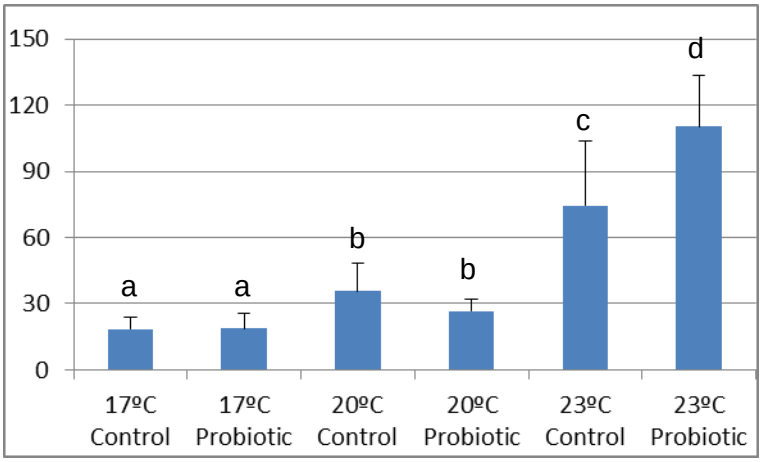

Chart 12: Amylase/Trypsin ratio mean values from all treatments. (ANOVA, *P*<0.05).

Trypsin / Chymotrypsin ratio

Trypsin / Chymotrypsin ratio (**Chart 13**) showed a significant difference between temperatures, being the 17 ºC treatments results significantly higher than the 23 ºC and 17 ºC, revealing a tendency in the lowest temperatures to act differently on the same protein sources (*P*-value < 0.01) ; (mean ± SD, n=12 fish.treatment^{-1}).

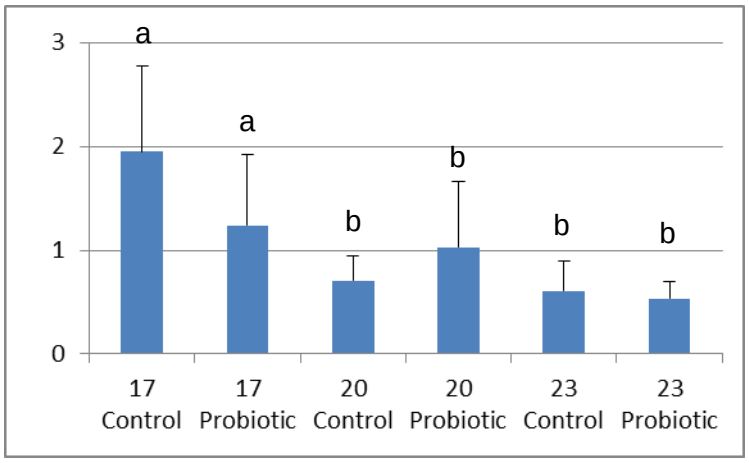

Chart 13: Trypsin enzymatic activities mean values from all treatments. (ANOVA, *P*<0.05).

Lipase

Lipase enzymatic activities (**Chart 14**) showed significantly higher results in the 23ºC treatment when compared with the other temperatures groups (*P*-value < 0.01). No difference was found between diets; (mean ± SD, n=12 fish.treatment^{-1}).

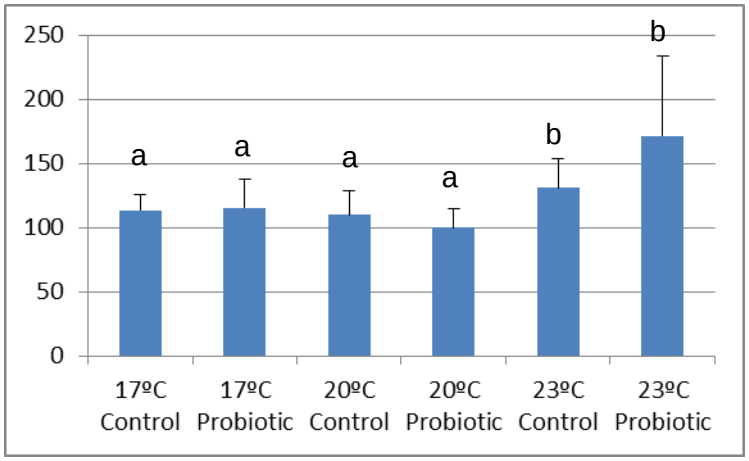

Chart 14: Lipase enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein^{-1}); (ANOVA, *P*<0.05).

4.5 Cumulative mortality

Cumulative mortalities (**Chart 15**) were noted for 10 days, starting with the stress event day (n=3 tanks.treatment^{-1}). Casualties differ between temperatures, having the lowest temperature (17 ºC) a greater damage on fish stock, specially the control treatments. Remaining temperatures did not seem to diverge considerately.

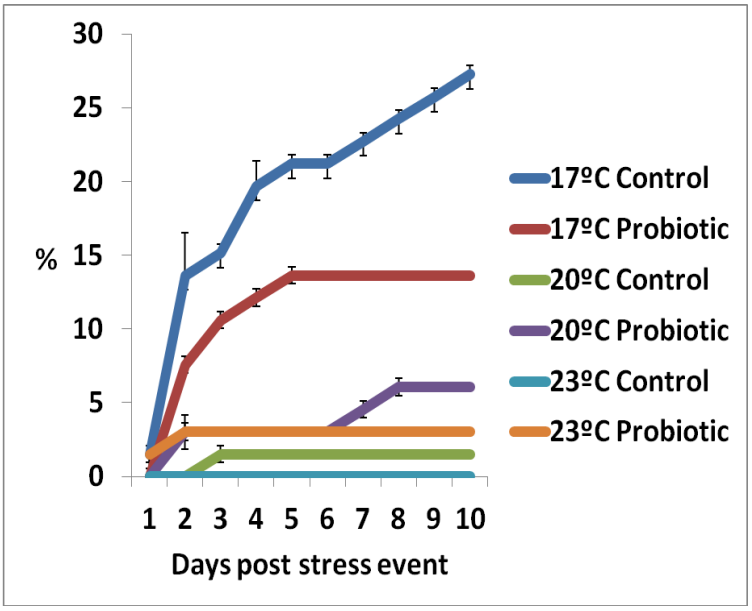

Chart 15: Cumulative mortalities results, referring to the ten days following the stress event.

5 Discussion

Growth performance

Temperature and dietary probiotic supplementation can play an important role in fish growth performance, since both have a modulatory effect on feeding, metabolism and feed digestibility. Within the tested probiotic blend, *Bacillus* and *Lactobacillus* genera seem to be the most correlated with growth improvement, either by influencing appetite, conversion ratio or reducing myostatin transcription (Lamari *et al.*, 2013, Carnevali *et al.*, 2006, Ringø & Gatesoupe, 1998, Ai *et al.*, 2011) a protein responsible for mitigating muscle growth and development. In the current study, growth was not significantly improved by dietary probiotic supplementation (**Chart 1**, **2** and **3**). It is plausible to infer that the use of pair-feeding method instead of the *ad libitum* feeding technique (Riddell *et al.*, 2010) may have restricted growth by the stimulation of appetite of probiotic supplementation. The temperature effect alone in growth results was expectable, as it fits the described optimum temperatures for seabass rearing (Person-Le Ruyet et al., 2004).

Humoral immune parameters

Probiotic supplementation and rearing temperature have shown to increase innate immune responses, often as a response to infection challenges (Touraki *et al.*, 2012, Aly *et al.*, 2008).

Lysozyme activity (**Table 4**) did not show any significant differences. Nevertheless, response to rearing temperature has been shown to be seasonal, but only when temperature decrease below 15 ºC, therefore our temperature range (17 – 23 ºC) might have evaded this decrease (Pascoli *et al.*, 2011, Valero *et al.*, 2014). Peroxidase activity lacked significant differences as well, but this has not shown any constant temperature influence in previous studies (Valero et al., 2014). Regarding probiotic influence, the characteristic reactive role in the immune system when facing a pathogen threat may have eluded significant differences.

Complement system alternative pathway results (**Table 4**) showed differences between temperatures, with the lower temperatures presenting higher activities, similar to what was observed in European seabass monitored during seasonal variation (Valero et al., 2014). Valero et al. (2014) observed that colder months tended to induce higher hemolytic activity in seabass. This response although similar to the cyprinidae family results (Collazos *et al.*, 1994), remains contradictory to what was found in seabream (Tort

et al., 1998). In our study, the lack of pathogen challenge trial may have reduced possible immune response differences between dietary groups.

Oxidative Stress: Enzymatic and non-enzymatic analyses

The effects of probiotic supplementation on antioxidant capacity has been described with and without pathogen challenge (Reyes-Becerril et al., 2011, Tovar *et al.*, 2002b). External factors alone such as rearing temperature are long-known for influencing oxidative stress indicators (Vinagre et al., 2012).

In our study, lipid peroxidation (LPO) (**Chart 6**), glutathione reductase (GR) (**Chart 7**) and total glutathione (TG) (**Chart 8**) showed significant differences between treatments. LPO results are in agreement with other studies (Vinagre et al., 2012), as it increases when temperature deviates from optimal range for the species Damage of lipid layers increases with the decrease in rearing temperature, as indicated by the malondialdehyde results. As a consequence, fish is less fit to cope to oxidative stress radicals. However no tendency for probiotic influence was found here.

TG showed to increase significantly by the probiotic supplementation, except at 23 ºC, in which TG values decreased with probiotic supplementation. Thus, the probiotic effect is temperature sensitive for TG content, indicating an inverse relationship at the highest temperature. When comparing the TG with the LPO results, it seems plausible to infer that the greater TG levels may have caused a decrease in the lipid damage. Here the protective role of glutathione appears more understandable. The fact that the 23 ºC probiotic treatment response shows a mitigated response in TG when compared to 23 ºC controls, which reveals a temperature differential effect on the TG levels. This may be due temperature modulation over the probiotic strains metabolic paths.

The effects of dietary probiotic supplementation on the antioxidant responses in aquatic organisms are poorly understood. Reyes et al. (2008b) observed an increase in superoxide dismutase activity in gilthead seabream and leopard grouper (*Mycteroperca rosacea*) fed *Debaryomyces hansenii*, but contrasted with Tovar-Ramírez et al. (2010) (Tovar-Ramírez et al., 2010)work with European seabass larvae. In this seabass work, yeast supplement did not affect catalase activity (CAT) but reduced glutathione peroxidase activity (GPX). In shrimp (*Litopenaeus vannamei*), GPX activity increased with dietary supplementation *Bacillus subtilis* (Shen *et al.*, 2010).

In fish reared at 20^{o}C, in contrast to fish reared at 17 ^{o}C and 23 ^{o}C, probiotic supplementation increased the glutathione reductase (GR) activity. As a consequence, more oxidized gluthatione (GSSG) are converted to reduced glutathione (GSH), a substrate necessary for the detoxification processes.

Digestive enzymes

Amylolytic (**Chart 9**) and lipolytic enzyme activities (**Chart 14**) increased with temperature, with no detectable diet effect. The effect of temperature on alpha-amylase was observed in Rohu (*Labeo rohita*) (Alexander *et al.*, 2011), where higher temperatures tended to increase carbohydrates digestibility.

The optimal temperature for lipids digestion varies according to the species optimal range, but in general, lipolytic enzyme shows higher activities at higher temperatures (*Oreochromis niloticus, Oncorhynchus mykiss*, *Gadus morhua,*(Kurtovic *et al.*, 2009). In the current study, probiotic treatment did not influence the amylase and lipase activities, contrasting to other studies. Askarian *et al.* (2011) observed an improvement of gut amylase and lipase activities in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fed lactic acid bacteria (*Bacillus* sp. and *Lactobacillus* sp.).

Trypsin activity (**Chart 10**) decreased with temperature, with no dietary influence. Temperature influence in trypsin activity has been shown to directly correlated until the 25 ºC in walking catfish (Ahmad *et al.*, 2014) and in yellowtail kingfish (*Seriola lalandi*) until the 22 ºC (Bowyer *et al.*, 2012). Some studies have also suggested trypsin as an indicator for digestibility and growth performance (Sunde *et al.*, 2004, Thongprajukaew et al., 2011). However this information is contradictory to our results. A possible theory for our proteases activities relays in the need for energy. As suboptimal temperatures turning more to protein deamination as energy source instead of carbohydrates, hence improving proteolytic activities, but misleading its enterpretation as a growth parameter. This metabolic tendency is known to occur when fish are reared outside of their optimal temperature range (FAO, 1978). Another explanation may come from the compensatory theory. A study in spotted wolffish (*Anarhichas minor*), reported lower trypsin activity at higher temperatures, suggesting a possible compensatory effect from the trypsin release in the intestine reared at optimal temperatures. Where proteolytic enzymes are over-expressed not because protein in being well converted, but as an attempt to surpass the mitigated growth (Savoie *et al.*, 2008). Another data that aids this energetic use for protein theory comes from our own results, in the protein efficiency ratio, where the lowest temperature treatments although with greater proteases activities, converted their consumed feed protein into growth less efficiently.

Chymotrypsin activity (**Chart 11**) has also been positively correlated with temperature. In catfish (*Clarias batrachus*), chymotrypsin activity increased at 25 ºC and then again at 35 ºC (Ahmad et al., 2014) but this tendency has been contradicted in Atlantic salmon, where chymotrypsin activity was greater at 6 ºC. Moreover, chymotrypsinogen is activated by trypsin, creating a limiting factor for chymotrypsin

activity. Chymotrypsin was significantly increased with probiotic treatment, whereas it decreased with increase in temperature, showing an interaction between temperature and diet. Our results are in agreement with (Zacarias-Soto *et al.*, 2011), which observed an increase in chymotrypsin activity in California Halibut, (*Paralichthys californicus*) fed *Pediococcus acidilactici* supplemented diet. However, chymotrypsin seems to have not yet been correlated with temperature and probiotic supplementation before.

Ratio between α-amylase and trypsin (A/T) (**Chart 12**), is often used as indicator of energy metabolism flexibility, showing the fish capability to use carbohydrates and sparing protein as energy source. In our study, fish reared at 23 ^{o}C outpaced fish reared at 17 ºC and 20 ºC, with a greater amylolitic capability, especially when fed supplemented probiotics. Highlighting a possible exocytosis amylase production from the probiotic strains as previously described (C De *et al.*, 2014). The lower carbohydrate digestion in the lower temperatures may have influenced as well the amino acid deamination for energy use.

The activity ratio of trypsin to chymotrypsin (T/C) (**Chart 13**), has been used as an indicator of growth performance, where lower T/C tend to indicate lack of growth stimulation (Rungruangsak-Torrissen, 2007). Such a decrease can be caused by a reduction of trypsin or an increase of chymotrypsin. In our study, the T/C decreased due to a decrease in trypsin, which may be correlated to trypsin expressions factors caused by feed limitation. Generally, well-fed fish have high trypsin activity, whereas starvation acts as a mitigating factor for this trypsin activity (Rungruangsak-Torrissen et al., 2006). Fish fed below satiation in the 23 ^{o}C groups, caused by the pair-feeding method may have their trypsin activity also influenced by the lack of satiation effect, hence decreasing their T/C ratio.

Cumulative mortality

Finally, post-stress cumulative mortality (**Chart 15**) were significantly higher in the 17ºC control group, showing an effect of temperature and dietary supplementation. Studies have shown strains like *Bacillus* spp. and *Lactobacillus* spp. to be capable of reducing mortalities of host when dealing with stress factors or direct pathogens challenges (Rollo *et al.*, 2006, Cerezuela *et al.*, 2012), and *Pediococcus spp*. and *Enterococcus spp*. by inhibiting pathogens infection (Rodriguez-Estrada *et al.*, 2013, Villamil *et al.*, 2010).

Interestingly, at 17 ^{o}C the alternative pathway complement system activity showed a more active pathogen attack complex, which were not translated into less mortality after

the *stress* challenge. This raises the question if a stimulated immune system can always be considered a good condition.

Future perspectives

As future perspective, considerations could be made regarding the probiotic installation success , as this may have been a major cause for the probiotic influence. For this, tecniques as denaturing gradient gel electriphoresis (DGGE), or pyrosequencing could be applied. Also, the probiotic potencial to improve carbohydrate and protein digestion could be better analysed, by quantifying the digestive enzymes activity in experimental diets with different levels of protein and carbohydratess. And finally, feed limitation over the 20ºC and 23ºC trials could be removed, by feeding with *ad libitum* method, thence possible influence of probiotic in daily feed intake could be analysed.

6 Conclusion

The use of dietary supplementation of probiotic blend was effective in modulating host anti-oxidant and digestive parameters. Nevertheless, no influences in the innate immune responses were observed. Probiotic supplementation was able to reduce fish mortality after a handling and crowding *stress* challenge in fish reared at 17 ^{o}C. An interaction between probiotic supplementation and rearing temperature was observed in the anti-oxidant responses and the digestive enzyme activity.

7 References

Ahmad, T., S. P. Singh, B. K. Khangembam, J. G. Sharma and R. Chakrabarti, 2014: Food consumption and digestive enzyme activity of *Clarias batrachus* exposed to various temperatures. *Aquaculture Nutrition,* **20,** 265-272

Ai, Q., H. Xu, K. Mai, W. Xu, J. Wang and W. Zhang, 2011: Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea*. *Aquaculture,* **317,** 155-161

Alexander, C., N. P. Sahu, A. K. Pal, M. S. Akhtar, S. Saravanan, B. Xavier and S. Munilkumar, 2011: Higher water temperature enhances dietary carbohydrate utilization and growth performance in *Labeo rohita* (Hamilton) fingerlings. *Journal of animal physiology and animal nutrition,* **95,** 642-652

Ali, E. H., M. Hashem and M. B. Al-Salahy, 2011: Pathogenicity and oxidative stress in Nile tilapia caused by *Aphanomyces laevis* and *Phoma herbarum* isolated from farmed fish. *Diseases of aquatic organisms,* **94,** 17-28

Alliot, E., A. Febvre, R. Metailler and A. Pastoureaud, Besoins nutritifs du bar (*Dicentrarchus labrax* l.) Etude du taux de protéine et du taux de lipide dans le régime. in Proceedings of the Colloque sur l'Aquaculture., 1974,

Alvarez-Pellitero, P., 2008: Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. *Veterinary immunology and immunopathology,* **126,** 171-198

Aly, S. M., Y. Abdel-Galil Ahmed, A. Abdel-Aziz Ghareeb and M. F. Mohamed, 2008: Studies on *Bacillus subtilis* and Lactobacillus acidophilus, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish & Shellfish Immunology,* **25,** 128-136

Angeles Esteban, M., A. Cuesta, A. Rodríguez and J. Meseguer, 2006: Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system. *Journal of pineal research,* **41,** 261-266

Applebaum, S., R. Perez, J. Lazo and G. Holt, 2001: Characterization of chymotrypsin activity during early ontogeny of larval red drum (*Sciaenops ocellatus*). *Fish Physiology and Biochemistry,* **25,** 291-300

Aragón-Axomulco, H., X. Chiappa-Carrara, L. Soto, G. Cuzon, L. Arena, C. Maldonado, R. Cárdenas and G. Gaxiola, 2012: Seasonal Variability in Trypsin and α-amylase Activities Caused by the Molting Cycle and Feeding Habits of Juvenile Pink Shrimp (Burkenroad, 1939). *Journal Of Crustacean Biology,* **32,** 89-99

Athel, C., 2004: Fundamentals of Enzyme Kinetics. *Portland Press,* **3rd edition**

Bagni, M., C. Civitareale, A. Priori, A. Ballerini, M. Finoia, G. Brambilla and G. Marino, 2007: Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (Dicentrarchus labrax) and sea bream (*Sparus aurata*). *Aquaculture,* **263,** 52-60

Baker, M. A., G. J. Cerniglia and A. Zaman, 1990: Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Analytical Biochemistry,* **190,** 360-365

Bates, J. M., J. Akerlund, E. Mittge and K. Guillemin, 2007: Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell host & microbe,* **2,** 371-382

Ben Ameur, W., J. de Lapuente, Y. El Megdiche, B. Barhoumi, S. Trabelsi, L. Camps, J. Serret, D. Ramos-Lopez, J. Gonzalez-Linares, M. R. Driss and M. Borras, 2012: Oxidative stress, genotoxicity and histopathology biomarker responses in mullet (*Mugil cephalus*) and sea bass (*Dicentrarchus labrax*) liver from Bizerte Lagoon (Tunisia). *Marine pollution bulletin,* **64,** 241-251

References

Bernfeld, P., 1951: Enzymes of starch degradation and synthesis. *Advances in enzymology and related subjects of biochemistry,* **12,** 379-428

Bielek, E., 1981: Developmental stages and localization of peroxidatic activity in the leucocytes of three teleost species (*Cyprinus carpio* L.; *Tinca tinca* L.; *Salmo gairdneri* Richardson). *Cell and tissue research,* **220,** 163-180

Biswas, G., H. Korenaga, R. Nagamine, S. Kawahara, S. Takeda, Y. Kikuchi, B. Dashnyam, T. Yoshida, T. Kono and M. Sakai, 2013: Elevated cytokine responses to *Vibrio harveyi* infection in the Japanese pufferfish (*Takifugu rubripes*) treated with *Lactobacillus paracasei* spp. *paracasei* (06TCa22) isolated from the Mongolian dairy product. *Fish & Shellfish Immunology,* **35,** 756-765.<Go to ISI>://WOS:000324511700016

Boshra, H., J. Li and J. O. Sunyer, 2006: Recent advances on the complement system of teleost fish. *Fish & Shellfish Immunology,* **20,** 239-262

Bowyer, J. N., J. G. Qin, L. R. Adams, M. J. S. Thomson and D. A. J. Stone, 2012: The response of digestive enzyme activities and gut histology in yellowtail kingfish (*Seriola lalandi*) to dietary fish oil substitution at different temperatures. *Aquaculture,* **368,** 19-28

Bradford, M., 1976: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry,* **72,** 248–254

Bragadóttir, M., 2001: Endogenous antioxidants in fish *Master of Science in food science Department of Food Science University of Iceland*

C De, B., D. K. Meena, B. K. Behera, P. Das, P. K. Das Mohapatra and A. P. Sharma, 2014: Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish physiology and biochemistry,* **40,** 921-971

Cahu, C. L. and J. L. Z. Infante, 1994: Early weaning of sea bass (*Dicentrarchus labrax*) larvae with a compound diet: Effect on digestive enzymes. *Comparative Biochemistry and Physiology Part A: Physiology,* **109,** 213-222

Can, E., I. Z. Kurtoglu, F. Benzer, M. Erisir, M. Kocabas, V. Kizak, M. Kayim and H. T. Celik, 2012: The Effects of Different Dosage of Kefir with Different Durations on Growth Performances and Antioxidant System in the Blood and Liver Tissues of Coruh Trout (*Salmo coruhensis*). *Turkish Journal of Fisheries and Aquatic Sciences,* **12,** 277-283

Carnevali, O., L. de Vivo, R. Sulpizio, G. Gioacchini, I. Olivotto, S. Silvi and A. Cresci, 2006: Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture,* **258,** 430-438

Castex, M., P. Lemaire, N. Wabete and L. Chim, 2009: Effect of dietary probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress status of shrimp *Litopenaeus stylirostris*. *Aquaculture,* **294,** 306-313

Cecchini, S. and M. Saroglia, 2002: Antibody response in sea bass (*Dicentrarchus labrax* L.) in relation to water temperature and oxygenation. *Aquaculture Research,* **33,** 607-613

Cerezuela, R., F. A. Guardiola, J. Meseguer and M. Esteban, 2012: Increases in immune parameters by inulin and *Bacillus subtilis* dietary administration to gilthead seabream (*Sparus aurata* L.) did not correlate with disease resistance to *Photobacterium damselae*. *Fish & shellfish immunology,* **32,** 1032-1040

Chiu, C. H., Y. K. Guu, C. H. Liu, T. M. Pan and W. Cheng, 2007: Immune responses and gene expression in white shrimp, *Litopenaeus vannamei*, induced by *Lactobacillus plantarum*. *Fish & shellfish immunology,* **23,** 364-377

Claiborne, A., 1985: Catalase activity. *In: Greenwald radical R. A. . CRC Handbok of Methods for Oxygen Radical Research.***,** 283-284

Collazos, M. a. E., C. Barriga and E. Ortega, 1994: Optimum conditions for the activation of the alternative complement pathway of a cyprinid fish (*Tinca tinca* L.). Seasonal variations in the titres. *Fish & Shellfish Immunology,* **4,** 499-506

Cowey, C. B. and C. Y. Cho, 1993: Nutritional requirements of fish. *Proceedings of the Nutrition Society,* **52,** 417-426

Cribb, A. E., J. S. Leeder and S. P. Spielberg, 1989: Use of a microplate reader in an assay of glutathione reductase using 5,5'-dithiobis(2-nitrobenzoic acid). *Analytical Biochemistry,* **183,** 195-196

Crocetta, F., 2014: European seabass (*Dicentrachus labrax*, Linnaeus 1758).http://www.fishbase.org/summary/63

De, B. C., D. K. Meena, B. K. Behera, P. Das, P. K. Das Mohapatra and A. P. Sharma, 2014: Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiology and Biochemistry,* **40,** 921-971

Defoirdt, T., P. Sorgeloos and P. Bossier, 2011: Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology,* **14,** 251-258

Dominguez, M., A. Takemura and M. Tsuchiya, 2005: Effects of changes in environmental factors on the non-specific immune response of Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Research,* **36,** 391-397

EC, 2003: Regulation (EC) 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal feed., *Official Journal of the European Union.*

EFSA, 2012: Scientific Opinion on the efficacy of Bactocell (*Pediococcus acidilactici*) when used as a feed additive for fish., *EFSA.*

Ellis, A., 1990: Lysozyme assays. *Techniques in fish immunology,* **1,** 101-103

Enes, P., S. Panserat, S. Kaushik and A. Oliva-Teles, 2006a: Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **143,** 89-96

Enes, P., S. Panserat, S. Kaushik and A. Oliva-Teles, 2006b: Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **145,** 73-81

Eroglu, A., Z. Dogan, E. G. Kanak, G. Atli and M. Canli, 2014: Effects of heavy metals (Cd, Cu, Cr, Pb, Zn) on fish glutathione metabolism. *Environ Sci Pollut Res***,** 1-9

Eshel, A., P. Lindner, P. Smirnoff, S. Newton and S. Harpaz, 1993: Comparative study of proteolytic enzymes in the digestive tracts of the european sea bass and hybrid striped bass reared in freshwater. *Comparative Biochemistry and Physiology Part A: Physiology,* **106,** 627-634

FAO, 1978: ADCP/REP/80/11 - Fish Feed Technology. *United nations development programme.*http://www.fao.org/docrep/x5738e/x5738e00.htm

FAOa, 2014: Species Fact Sheets - *Dicentrarchus labrax* (Linnaeus, 1758).http://www.fao.org/fishery/species/2291/en

FAOb, 2014: Cultured Aquatic Species Information Programme *Dicentrarchus labrax* (Linnaeus, 1758).http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en

FAOc, 2012: *Dicentrarchus labrax* Global production.http://data.fao.org/dataset-data-filter?entryId=033ae7cf-b322-4dc5-8dfe-140140c56008&tab=data&type=Dimensionmember&uuidResource=65017a3d-81ba-4690-aa6e-52166bc26aaf

Finkel, T. and N. J. Holbrook, 2000: Oxidants, oxidative stress and the biology of ageing. *Nature,* **408,** 239-247

Fishbase, 2014: European seabass - *Dicentrarchus labrax* (Linnaeus, 1758).http://www.fishbase.org/summary/Dicentrarchus-labrax.html

Fournier, V., M. F. Gouillou-Coustans and S. J. Kaushik, 2000: Hepatic ascorbic acid saturation is the most stringent response criterion for determining the vitamin C requirement of juvenile European sea bass (*Dicentrarchus labrax*). *The Journal of nutrition,* **130,** 617-620

Fredrickson, A. and G. Stephanopoulos, 1981: Microbial competition. *Science,* **213,** 972-979

Geay, F., S. Ferraresso, J. L. Zambonino-Infante, L. Bargelloni, C. Quentel, M. Vandeputte, S. Kaushik, C. L. Cahu and D. Mazurais, 2011: Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass

(*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. *Biomedcentral Genomics,* **12,** 522

Gioacchini, G., E. Giorgini, I. Olivotto, F. Maradonna, D. L. Merrifield and O. Carnevali, 2014: The Influence of Probiotics on Zebrafish *Danio Rerio* Innate Immunity and Hepatic Stress. *Zebrafish,* **11,** 98-106

Gismondo, M. R., L. Drago and A. Lombardi, 1999: Review of probiotics available to modify gastrointestinal flora. *The International Journal of Antimicrobial Agents,* **12,** 287-292

Gravato, C. and L. Guilhermino, 2009: Effects of Benzo(a)pyrene on Seabass (*Dicentrarchus labrax* L.): Biomarkers, Growth and Behavior. *Human and Ecological Risk Assessment,* **15,** 121-137

Grosell, M., A. P. Farrell and C. J. Brauner, 2010: *Fish Physiology: The Multifunctional Gut of Fish: The Multifunctional Gut of Fish*. Academic Press,

Guerriero, G., A. Di Finizio and G. Ciarcia, 2002: Stress-induced changes of plasma antioxidants in aquacultured sea bass, *Dicentrarchus labrax*. *omparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **132,** 205-211

Habig, W. H., M. J. Pabst and W. B. Jakoby, 1974: Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *The journal Chemistry & Biology,* **249,** 7130-7139

Hagi, T., D. Tanaka, Y. Iwamura and T. Hoshino, 2004: Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture,* **234,** 335-346

Henry, M., M. Alexis, E. Fountoulaki, I. Nengas and G. Rigos, 2009: Effects of a natural parasitical infection (*Lernanthropus kroyeri*) on the immune system of European sea bass, *Dicentrarchus labrax* L. *Parasite immunology,* **31,** 729-740

Hidalgo, F. and E. Alliot, 1988: Influence of water temperature on protein requirement and protein utilization in juvenile sea bass, *Dicentrarchus labrax*. *Aquaculture,* **72,** 115-129

Hidalgo, F., E. Alliot and H. Thebault, 1987: Methionine-and cystine-supplemented diets for juvenile sea bass (*Dicentrarchus labrax*). *Aquaculture,* **64,** 209-217

Hjelmeland, K., I. Huse, T. Jorgensen, G. Molvik and J. Raa, 1984: Trypsin and trypsinogen as indices of growth and survival potential of cod (*Gadus morhua* L.) larvae. *The propagation of cod***,** 189-202

Ibrahim, F., A. C. Ouwehand and S. J. Salminen, 2004: Effect of temperature on in vitro adhesion of potential fish probiotics. *Microbial Ecology in Health and Disease,* **16,** 222-227

ICES, 2013: Report of the Working Group on Report on Assessment of New MoU Species (WGNEW).ICES ADVISORY COMMITTEE. http://archimer.ifremer.fr/doc/00019/13054/10009.pdf

INE, 2013: Instituto Nacional de Estatística.http://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_main

Infante, J. L. Z. and C. L. Cahu, 1999: High dietary lipid levels enhance digestive tract maturation and improve *Dicentrarchus labrax* larval development. *The Journal of nutrition,* **129,** 1195-1200

Irianto, A. and B. Austin, 2002: Probiotics in aquaculture. *Journal of fish diseases,* **25,** 633–642

Jaica, 2014. Japan institute of the control of aging.http://www.jaica.com/e/products_protein_2br_y_ab.html

Jollès, P. and J. Jollès, 1984: What's new in lysozyme research? *Molecular and cellular biochemistry,* **63,** 165-189

Katzenback, B. A., F. Katakura and M. Belosevic, 2012: *Regulation of Teleost Macrophage and Neutrophil Cell Development by Growth Factors and Transcription Factors*.

Kaushik, S. J., 1998: Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. *Aquatic Living Resources,* **11,** 355-358

Klebanoff, S. J., 1968: Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *Journal of bacteriology,* **95,** 2131-2138

Koumans-van Diepen, J. C. E., 1993: Characterisation of fish leucocytes: an immunocytochemical and functional study in carp *(Cyprinus carpio L.)*. Landbouwuniversiteit te Wageningen,

Kurtovic, I., S. N. Marshall, X. Zhao and B. K. Simpson, 2009: Lipases from Mammals and Fishes. *Reviews in Fisheries Science,* **17,** 18-40

Kuz'mina, V. V., 1996: Influence of age on digestive enzyme activity in some freshwater teleosts. *Aquaculture,* **148,** 25-37

Lamari, F., M. Castex, T. Larcher, M. Ledevin, D. Mazurais, A. Bakhrouf and F.-J. Gatesoupe, 2013: Comparison of the effects of the dietary addition of two lactic acid bacteria on the development and conformation of sea bass larvae, *Dicentrarchus labrax*, and the influence on associated microbiota. *Aquaculture,* **376–379,** 137-145

Livingstone, D. R., 2003: Oxidative stress in aquatic organisms in relation to pollution and aquaculture. *Revue De Medecine Veterinaire,* **154,** 427-430

Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall, 1951: Protein measurement with the Folin phenol reagent. *The Journal of Chemistry & Biology,* **193,** 265-275

Lushchak, V. I., 2011: Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology,* **101,** 13-30

Maine.University, 2014: The Serine Proteases - I.http://chemistry.umeche.maine.edu/CHY431/Peptidase.html

Martinez Cruz, P., A. L. Ibanez, O. A. Monroy Hermosillo and H. C. Ramirez Saad, 2012: Use of probiotics in aquaculture. *International Scholarly Research Notices Microbiology,* **2012**

Mohandas, J., J. J. Marshall, G. G. Duggin, J. S. Horvath and D. J. Tiller, 1984: Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney. Possible implications in analgesic nephropathy. *Biochemical pharmacology,* **33,** 1801-1807

Mohapatra, S., T. Chakraborty, V. Kumar, G. DeBoeck and K. N. Mohanta, 2013: Aquaculture and stress management: a review of probiotic intervention. *Journal of animal physiology and animal nutrition,* **97,** 405-430

Mohapatra, S., T. Chakraborty, A. K. Prusty, P. Das, K. Paniprasad and K. N. Mohanta, 2012: Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. *Aquaculture Nutrition,* **18,** 1-11

Nakatsuji, T. and R. L. Gallo, 2012: Antimicrobial peptides: old molecules with new ideas. *Journal of investigative dermatology,* **132,** 887-895

Namrata, C., 2013: Digestion of Lipids - A quick revision.http://www.namrata.co/digestion-of-lipids-a-quick-revision/

Nayak, S. K., 2010a: Probiotics and immunity: a fish perspective. *Fish and Shellfish Immunology,* **29,** 2-14

Nayak, S. K., 2010b: Role of gastrointestinal microbiota in fish. *Aquaculture Research,* **41,** 1553-1573

Ogiwara, K. and T. Takahashi, 2007: Specificity of the medaka enteropeptidase serine protease and its usefulness as a biotechnological tool for fusion-protein cleavage. *Proceedings of the National Academy of Sciences,* **104,** 7021-7026

Ohkawa, H., N. Ohishi and K. Yagi, 1979: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry,* **95,** 351-358

Olsen, R. E. and R. J. Henderson, 1997: Muscle fatty acid composition and oxidative stress indices of Arctic charr, *Salvelinus alpinus* (L.), in relation to dietary polyunsaturated fatty acid levels and temperature. *Aquaculture Nutrition,* **3,** 227-238

Olsen, R. E., K. Sundell, T. M. Mayhew, R. Myklebust and E. Ringø, 2005: Acute stress alters intestinal function of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture,* **250,** 480-495

Papoutsoglou, E. and A. Lyndon, 2003: Distribution of α amylase along the alimentary tract of two Mediterranean fish species, the parrotfish *Sparisoma cretense* L. and the stargazer, *Uranoscopus scaber* L. *Mediterranean Marine Science,* **4,** 115-124

Pascoli, F., G. S. Lanzano, E. Negrato, C. Poltronieri, A. Trocino, G. Radaelli and D. Bertotto, 2011: Seasonal effects on hematological and innate immune parameters in sea bass *Dicentrarchus labrax*. *Fish and Shellfish Immunology,* **31,** 1081-1087

Pascual, P., J. R. Pedrajas, F. Toribio, J. Lopez-Barea and J. Peinado, 2003: Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). *Chemico-biological interactions,* **145,** 191-199

Paul, H., 2012: Enzymatic Digestion of Polysaccharides (Part II). Applications Dept, BioTek Instruments, Inc.http://www.biotek.com/resources/articles/enzymatic-digestion-of-polysaccharides-2.html

Pereira, L. F., J. Gonçalves, A. F. Domingues, M. D. Peixoto, S. Braga, K. Sansuwan, G. Santos and R. O. A. Ozorio, 2014: Dietary probiotic supplementation improved gut amylase to trypsin ratio in European seabass reared at different temperatures and survival after handling stress. *Frontiers in Marine Science*. http://www.frontiersin.org/Journal/FullText.aspx?f=45&name=marine_science&ART_DOI=10.3389/conf.FMARS.2014.02.00016

Peres, H. and A. Oliva-Teles, 2002: Utilization of raw and gelatinized starch by European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture,* **205,** 287-299

Person-Le Ruyet, J., K. Mahé, N. Le Bayon and H. Le Delliou, 2004: Effects of temperature on growth and metabolism in a Mediterranean population of European sea bass, *Dicentrarchus labrax*. *Aquaculture,* **237,** 269-280

Pineiro, M. and C. Stanton, 2007: Probiotic Bacteria: Legislative Framework— Requirements to Evidence Basis. *The Journal of Nutrition,* **137,** 850S-853S

Quade, M. J. and J. A. Roth, 1997: A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. *Veterinary Immunology & Immunopathology,* **58,** 239-248

Rabanal, H. R., 1988: *History of aquaculture*. ASEAN/UNDP/FAO Regional Small-Scale Coastal Fisheries Development Project, Manila.http://www.fao.org/docrep/field/009/ag158e/ag158e02.htm

Reyes-Becerril, M., D. Tovar-Ramírez, F. Ascencio-Valle, R. Civera-Cerecedo, V. Gracia-López, V. Barbosa-Solomieu and M. Á. Esteban, 2011: Effects of dietary supplementation with probiotic live yeast *Debaryomyces hansenii* on the immune and antioxidant systems of leopard grouper Mycteroperca rosacea infected with *Aeromonas hydrophila*. *Aquaculture Research,* **42,** 1676-1686

Riddell, J. B., A. J. Gallegos, D. L. Harmon and K. R. McLeod, 2010: Addition of a *Bacillus* based probiotic to the diet of preruminant calves: Influence on growth, health, and blood parameters. *International Journal of Applied Research in Veterinary Medicine,* **8,** 78-85

Ringø, E. and F.-J. Gatesoupe, 1998: Lactic acid bacteria in fish: a review. *Aquaculture,* **160,** 177-203

Roberts, R. J., 2012: *Fish pathology*. John Wiley & Sons,

Rodriguez-Estrada, U., S. Satoh, Y. Haga, H. Fushimi and J. Sweetman, 2013: Effects of Inactivated *Enterococcus faecalis* and Mannan Oligosaccharide and Their Combination on Growth, Immunity, and Disease Protection in Rainbow Trout. *North American Journal of Aquaculture,* **75,** 416-428

Rollo, A., R. Sulpizio, M. Nardi, S. Silvi, C. Orpianesi, M. Caggiano, A. Cresci and O. Carnevali, 2006: Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiology and Biochemistry,* **32,** 167-177

Rungruangsak-Torrissen, K., 2007: Digestive efficiency, growth and qualities of muscle and oocyte in atlantic salmon (*Salmo salar* L.) fed diets with krill meal as an alternative protein source. *Journal of Food Biochemistry,* **31,** 509-540

Rungruangsak-Torrissen, K., R. Moss, L. H. Andresen, A. Berg and R. Waagbo, 2006: Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (*Salmo salar* L.). *Fish Physiology and Biochemistry,* **32,** 7-23

Rungruangsak-Torrissen, K. and A. Sundby, 2000: Protease activities, plasma free amino acids and insulin at different ages of Atlantic salmon (*Salmo salar* L.) with genetically different trypsin isozymes. *Fish Physiology and Biochemistry,* **22,** 337-347

Rungruangsak Torrissen, K. and R. Male, 2000: Trypsin isozymes: development, digestion and structure. *Seafood Enzymes,* **97,** 215-269

Sarropoulou, E., J. M. Fernandes, K. Mitter, A. Magoulas, V. Mulero, M. P. Sepulcre, A. Figueras, B. Novoa and G. Kotoulas, 2010: Evolution of a multifunctional gene: The warm temperature acclimation protein Wap65 in the European seabass *Dicentrarchus labrax*. *Molecular phylogenetics and evolution,* **55,** 640-649

Savoie, A., N. R. Le Francois, C. Cahu and P. U. Blier, 2008: Metabolic and digestive activity profiles of newly hatched spotted wolffish (*Anarhichas minor* Olafsen): effect of temperature. *Aquaculture Research,* **39,** 382-389

Scapigliati, G., N. Romano, F. Buonocore, S. Picchietti, M. Baldassini, D. Prugnoli, A. Galice, S. Meloni, C. Secombes and M. Mazzini, 2002: The immune system of sea bass, *Dicentrarchus labrax*, reared in aquaculture. *Developmental & Comparative Immunology,* **26,** 151-160

Sengupta, P. and P. Garrity, 2013: Sensing temperature. *Curr Biol,* **23,** R304-307

Sevcikova, M., H. Modra, A. Slaninova and Z. Svobodova, 2011: Metals as a cause of oxidative stress in fish: a review. *Veterinarni Medicina,* **56,** 537-546

Shen, W. Y., L. L. Fu, W. F. Li and Y. R. Zhu, 2010: Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). *Aquaculture Research,* **41,** 1691-1698

Smith, L. S., 1989: Digestive functions in teleost fishes. *Fish nutrition,* **2,** 331-421

SOFIA, 2014: The state of world fisheries and aquaculture. *Food and Agriculture Organization of the United Nations.,*

Sun, Y. Z., H. L. Yang, R. L. Ma and W. Y. Lin, 2010: Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. *Fish and Shellfish Immunology,* **29,** 803-809

Sun, Y. Z., H. L. Yang, R. L. Ma, K. Song and J. S. Li, 2012: Effect of Lactococcus lactis and Enterococcus faecium on growth performance, digestive enzymes and immune response of grouper *Epinephelus coioides*. *Aquaculture Nutrition,* **18,** 281-289.http://dx.doi.org/10.1111/j.1365-2095.2011.00894.x

Sunde, J., S. A. Eiane, A. Rustad, H. B. Jensen, J. Opstvedt, E. Nygard, G. Venturini and K. Rungruangsak-Torrissen, 2004: Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Aquaculture Nutrition,* **10,** 261-277

Sunyer, J. O., E. Gómez, L. Tort, V. Navarro and J. Quesada, 1995: Physiological responses and depression of humoral components of the immune system in gilthead sea bream (*Sparus aurata*) following daily acute stress. *Canadian Journal of Fisheries and Aquatic Sciences,* **52,** 2339-2346

Taoka, Y., H. Maeda, J.-Y. Jo, M.-J. Jeon, S. C. Bai, W.-J. Lee, K. Yuge and S. Koshio, 2006: Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Science,* **72,** 310-321

Thongprajukaew, K., U. Kovitvadhi, S. Kovitvadhi, P. Somsueb and K. Rungruangsak-Torrissen, 2011: Effects of different modified diets on growth, digestive enzyme activities and muscle compositions in juvenile Siamese fighting fish (*Betta splendens* Regan, 1910). *Aquaculture,* **322–323,** 1-9

Tibaldi, E. and D. Lanari, 1991: Optimal dietary lysine levels for growth and protein utilisation of fingerling sea bass (*Dicentrarchus labrax* L.) fed semipurified diets. *Aquaculture,* **95,** 297-304

Torrecillas, S., A. Makol, M. Caballero, D. Montero, L. Robaina, F. Real, J. Sweetman, L. Tort and M. Izquierdo, 2007: Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish & Shellfish Immunology,* **23,** 969-981

Torrissen, K. R., 1984: Characterization of protéases in digestive-tract of atlantic salmon (*Salmo salar*) in comparison with rainbow throut (*Salmo gairdneri*). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,* **77,** 669-674

Tort, L., J. Rotllant and L. Rovira, 1998: Immunological suppression in gilthead sea bream (*Sparus aurata*) of the North-West Mediterranean at low temperatures. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **120,** 175-179

Touraki, M., G. Karamanlidou, P. Karavida and K. Chrysi, 2012: Evaluation of the probiotics *Bacillus subtilis* and *Lactobacillus plantarum* bioencapsulated in Artemia nauplii against vibriosis in European sea bass larvae (*Dicentrarchus labrax*, L.). *World Journal of Microbiology and Biotechnology,* **28,** 2425-2433

Tovar-Ramírez, D., D. Mazurais, J. F. Gatesoupe, P. Quazuguel, C. L. Cahu and J. L. Zambonino-Infante, 2010: Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture,* **300,** 142-147

Tovar, D., J. Zambonino, C. Cahu, F. Gatesoupe, R. Vázquez-Juárez and R. Lésel, 2002a: Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture,* **204,** 113-123

Tovar, D., J. Zambonino, C. Cahu, F. J. Gatesoupe, R. Vázquez-Juárez and R. Lésel, 2002b: Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture,* **204,** 113-123.http://www.sciencedirect.com/science/article/pii/S0044848601006500

UIC, 2014: The immune system. The university of Illinois at Chicago.http://www.uic.edu/classes/bios/bios100/lecturesf04am/lect23.htm

Valero, Y., A. García-Alcázar, M. Á. Esteban, A. Cuesta and E. Chaves-Pozo, 2014: Seasonal variations of the humoral immune parameters of European sea bass (*Dicentrarchus labrax* L.). *Fish & Shellfish Immunology,* **39,** 185-187

Vázquez, F. J. S. and J. A. Muñoz-Cueto, 2014: *Biology of European Sea Bass*. CRC Press,

Vilà, B., E. Esteve-Garcia and J. Brufau, 2010: Probiotic micro-organisms: 100 years of innovation and efficacy; modes of action. *World's Poultry Science Journal,* **66,** 369-380

Villamil, L., A. Figueras, M. Planas and B. Novoa, 2010: *Pediococcus acidilactici* in the culture of turbot (*Psetta maxima*) larvae: Administration pathways. *Aquaculture,* **307,** 83-88

Vinagre, C., D. Madeira, L. Narciso, H. N. Cabral and M. Diniz, 2012: Effect of temperature on oxidative stress in fish: Lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. *Ecological Indicators,* **23,** 274-279

Vine, N. G., W. D. Leukes and H. Kaiser, 2006: Probiotics in marine larviculture. *FEMS Microbiology Reviews,* **30,** 404-427

Vine, N. G., W. D. Leukes, H. Kaiser, S. Daya, J. Baxter and T. Hecht, 2004: Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *Journal of Fish Diseases,* **27,** 319-326

Wang, Y. B., Z. Q. Tian, J. T. Yao and W. F. Li, 2008: Effect of probiotics, Enteroccus faecium, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture,* **277,** 203-207.

Webster, C. D. and C. Lim, 2002: *Nutrient requirements and feeding of finfish for aquaculture*. Cabi,

Winkler, U. K. and M. Stuckmann, 1979: Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology,* **138,** 663-670

Worthington.corp, 2014: Trypsin.http://www.worthington-biochem.com/try/default.html

Zacarias-Soto, M., J. P. Lazo and M. T. Viana, 2011: Effect of Three Probiotics Administered Through Live Feed on Digestive Enzyme Activity in California Halibut, *Paralichthys californicus*, Larvae. *Journal of the World Aquaculture Society,* **42,** 321-331

Zhao, H., D. Han, S. Xie, X. Zhu and Y. Yang, 2009: Effect of water temperature on the growth performance and digestive enzyme activities of Chinese longsnout catfish (*Leiocassis longirostris* Günther). *Aquaculture Research,* **40,** 1864-1872

Zong, W. X. and C. B. Thompson, 2006: Necrotic death as a cell fate. *Genes & development,* **20,** 1-15

8 Attatchments

8.1 Laboratory analysis procedures

Complement system – (Sunyer et al., 1995)

The procedure goes as follows: firstly the sample of rabbit red blood cells must be cleaned with physiological serum (NaCl 0.9%) in a 1:4 ratio (blood:serum), and a quick 5 minutes centrifugation at 2000 g forces. From this we should collect a clear pink solution with about $2{,}8x10^8$ cell/ml (RaRBC), using a 1:200 dilution for counting cells under a microscope at a total amplification of 100 times. From here, we should start to measure the correct dilution required for the 50% hemolysis, by adding 10ul of our plasma sample, and a variable amount of rabbit's blood solution ate the previously mentioned concentration to the 96-well microplate. The testes dilutions would be of 1:16, 1:32, :48, 1:64, 1:96. This incubation would go for 100 minutes and stopped with a solution composed of: 0.1% gelatin, 5mM sodium barbiturate, 0.13mM sodium chloride and 20mM EDTA, with a final pH calibration for 7.3.

Finally the microplates would be centrifuged at 122 g forces and from this, 150ul would be pippeted to a new microplate and the lysed cells measured at the absorbance of 414nm wavelength. The alternative complement pathway (ACH50) units were defined as the concentration of serum giving 50% haemolysis of RaRBC. All analysis were conducted by triplicates.

Peroxidase - (Quade & Roth, 1997)

To estimate the plasmatic peroxidase content, initially 135 ul of a stabilizing environment of Hank's Balanced Salt Solution, (HBSS Ca2+ free) solution was added to a flat bottom 96-well microplate, then 15ul of plasma sample, 50ul of tetremethylbenzidine hydrochloride (TMB) and 50ul of hydrogen peroxide (H2O2), this would allow the reaction to occur. Later, 50ul of a 2mM of sulfuric acid (H2SO4) would stop the reaction. The actual measurement would be made by spectrophotometry at 450 nm wavelength. The principle behind the reaction is, TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes, such as the exocytosis myeloperoxidase and eosinophilic peroxidase. The reaction when halted by addition of sulfuric acid turns TMB yellow. Finally, the colour may be read at 450 nm. Final unit is presented as enzymatic units, with one enzymatic being defined as the amount producing an absorbance change of 1.

Lysozyme - (Ellis, 1990)

This procedure starts with the preparation of a 0.5 mg/ml of *Micrococus lysodeikticusa* in a 0.05M sodium phosphate dibasic (Na2HPO4) solution. This bacteria solution would be used as target subtract for the lysozyme samples. Actual measurement would come from comparison with 15 known concentrations of Hen egg-white lysozyme (HEWL), from 1mg/ml to 0,0049ug/ml. A reaction mix of 15ul plasma sample and 250ul of bacterial suspension would then be read at 450nm wavelength in 2 timings, 30 seconds and 270 seconds. From these absorbances values the conversion to enzymatic units (EU) would be made by the convention of 1 EU = 0.001 absorbance units / minute.

Protein quantification - (Bradford, 1976)

Initially a standard curve of 250 µl BioRad® with 10ul of a known concentration of bovine gamma globulin (0; 0.2; 0.5 and 1 $mg.ml^{-1}$) was created. Followed by a 15 minutes incubation reaction in the dark to avoid light damage, and final reading at 600 nm, we were then able to calculate the concentration of our samples placed under the same conditions.

Lipid peroxidation - (Ohkawa et al., 1979)

The reaction complex is composed of: 150 µl of liver homogenate, 500 µl of TBA (Thiobarbituric acid), 500 ul of TCA (Trichloroacetic acid), 400 µl of Tris-HCl (Trisaminometane) at 60mM with 0.1 mM DTPA (diethylene triamine pentaacetic acid). After an incubation period of 60 minutes at 100 ºC, a centrifugation of 11500 g forces at 25 ºC for 5 minutes would be made. Only then the samples would be read at 535nm. The principle of this analysis is: the MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (100°C) and acidic conditions (TCA) can be measured colorimetrically at 535 nm. With the absorbance of this measurement and the extinction coefficient of the MDA-TBA complex of 1.56×10^5 $M^{-1}.cm^{-1}$, the actual concentration of the lipid peroxidation product (MDA), can then be quantified. Finally, units are presented as nmoles of MDA formed.mg $protein^{-1}$.

Catalase - (Claiborne, 1985)

The reaction complex was based on 15 µl of the PMS, 135 µl of K-phosphate buffer at 0.05 M (pH 7) and 150 µl of peroxide hydrogen at 0.03 M. The peroxide hydrogen concentration would then be pursuit by spectofotometry at 240 nm for the first minute. This absorbance together with the peroxide hydrogen extinction coefficient (40 M^{-}

^{1}. cm^{-1}) allowed the calculation of the catalase activity slope. Final unit is presented as hydrogen peroxide µmol.min^{-1}.protein mg^{-1}.

Glutathione s-transferase - (Habig et al., 1974)

For this reaction, 100 µl of PMS was added to 4.95 ml of phosphate buffer (0.1M pH 6.5), 900 µL of reduced glutathione (GSH) at 10 mM and 150 µL of CDNB (1-chloro-2,4-dinitrobenzene) at 10 mM. Spectofotometer readings were made at 340 nm wavelength every 20 seconds for 5 minutes after all reagents were combined.The enzyme glutathione-S-transferase (GST) conjugates CDNB (substrate) with glutathione (GSH) originating a complex The formation of this compound (ε =9.6 $mM^{-1}.cm^{-1}$) can be monitored at 340 nm, registering an increase in the absorbance over time. This is finally expressed in µmol GSH-CDNB conjugate formed.min^{-1}.protein mg^{-1}.

Glutathione peroxidase - (Mohandas et al., 1984)

This requires the peptide GSH and peroxide hydrogen to recreated reaction mix composed of: 840 µl of K-Phosphate 0.05M (pH 7.0) with EDTA 1 mM and Sodium azide (1 mM) and GR at 1U/ml; 50 µL GSH 4 mM, 50 µL NADPH (Nicotinamide adenine dinucleotide phosphate) 0.8 mM, 10 µL H2O2 0.5 mM and finally 50 µL of PMS. Measures were made at 340 nm for 30 seconds. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GPX activity, since GPX is the rate limiting factor of the coupled reactions.

Glutathione reductase - (Cribb et al., 1989)

In this method a 950 µl solution with 0.0172g of NADPH, 0.0654g of GSSG and 0.0196g of DTPA is mixed. Readings were then made at 340 nm wave length during 1 minute. The rate of increase in absorbance is directly proportional to the amount of glutathione reductase in the sample. Then measured at 340 nm during the oxidation of NADPH to NADP+ is indicative of GR activity.

Total Glutathione - (Baker et al., 1990)

Total glutathione (TG) required a previous treatment, where 10 µl of ultra pure water were added to 200 µl PMS and left to incubate for 1 hour at 25 ºC. After this, 200 µl of 12% pure TCA (Trichloroacetic acid) were added and another incubation of 1 hour at 4ºC, followed by a centrigugation at 10000 g forces for 5 minutes. The reaction per se, included 150 µL of treated PMS, 2.1 mL Na-K phosphate (0.1M pH 8.0), 75 µL of NADPH, 100 µL of DTNB and 25 µL of GR. Reading was made at 412nm wave length for 1 minute.

Calculations of the formulation of TNB (5-Mercapto-2-nitrobenzoic acid); (ε =14.1x10^3 M^{-1}.cm^{-1}).

Enzyme extraction and protein quantification - (Rungruangsak-Torrissen, 2007, Lowry et al., 1951)

Intestine was homogenized in in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:1 w/v) using a homogenizer/blender, followed by centrifugation at 15000 g forces for 30 minutes at 4 ºC, and finally prepared the required samples for protein and digestive enzymes activity quantification. The quantification was made following the folin phenol method. A volume of 25 µl enzyme extract was combined with 750 of 0.05% Na2CO3, 2 mM NaOH, 0.5 % CuS04 and 1 % sodium or potassium tartrate with 50% folin solution. Left incubate for 30 minutes in dark and finally measured at 700 nm wave length. Comparison of values was made with a grade of known values of bovine serum albumin concentrations.

A-Amylase - (Bernfeld, 1951)

A 5% Starch solution was used as substrate, added to 37.5 µl of NaCl (20 mM) and 125 µl of enzyme extract, finally left to incubate at 37 ºC for 15 minutes. Reaction was stopped with the addition of 2.5 ml water and maltose stained with 250 µl DNS, followed by 5 minutes at 100 ºC. The produced maltose was determined by measuring the changes in absorbance at 540 nm, as DNS-Maltose complex turned from yellow to red-brown color. Calculations were made in comparison to a grade of 11 known maltose concentrations (0 to 50 umol.ml^{-1}).

Trypsin - (Torrissen, 1984)

Trypsin activity was determined by incubating 100 µl of enzyme extract with 700 µl of trypsin substrate [1.25 mM benzoyl-L-arginine-*p*-nitroanilide (dissolved with dimethylformamide (5% final concentration) before made up to final volume with 0.2 M Tris-HCl buffer pH 8.4)] at 25 °C for 10 min. The reaction was stopped by the addition of 800 µl 30% acetic acid. The production of nitroaniline was measured spectrophotometrically at 410 nm, and compared with a nitroaniline standard curve.

Chymotrypsin - (Rungruangsak-Torrissen & Sundby, 2000)

Chymotrypsin activity was determined by incubating 100 µl enzyme extract with 700 µl of chymotrypsin substrate [0.1 mM succinyl-Ala-Ala-Ala-Pro-Phe-*p*-nitroanilide (dissolved with dimethylformamide (5% final concentration) before made up to final

volume with 0.2 M Tris-HCl buffer pH 8.4)] at 25 °C for 10 min. The reaction was stopped and measured at 410 nm in the same way as trypsin.

Lipase - (Winkler & Stuckmann, 1979)

Lipase activity assay was performed using ρ- nitrophenyl substrate as described by Winkler and Stuckmann (Winkler & Stuckmann, 1979). The reaction mixture consisted of 0.01 M *p*-Nitrophenyl palmitate, 800 µl of 0.2 M buffer (0.1M Sodium carbonate pH 9) and 10 µl of enzyme extract. This was left to incubate for 15 minutes at 30 ºC. The reaction was stopped with 250 µl 1 M Na_2CO_3. Followed by a 10000 g forces centrifugation for 15 minutes and reading at 410 nm. Calculations were made according to a p-nitrophenol standard curve.

8.2 Presentations

IJUP – Meeting of young researchers at University of Porto

Februart 12th – Oral Presentation

Certifica-se que Luis Ferrreira Pereira esteve presente no IJUP'14 – *7º Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 12, 13 e 14 de fevereiro de 2014, na Reitoria da Universidade do Porto, tendo apresentado a comunicação oral "Growth performance, antioxidant and immune responses in European seabass fed dietary probiotic supplementation under different rearing temperatures.".

Pela Comissão Organizadora

(O Vice-Reitor, Prof. Doutor Jorge Gonçalves)

IMMR'14 – International Meeting on Marine Research 2014

July 11th – Oral Presentation

Published in *Frontiers in Marine Science Journal*

DOI: 10.3389/conf.FMARS.2014.02.00016 **- (Pereira *et al.*, 2014)**

CERTIFICATE OF PARTICIPATION

Oral Communication

This is to certify that,

Luís F. Pereira

attended to the International Meeting on Marine Research 2014 [IMMR'14] held at Peniche (Portugal), on the 10th and 11th of July, and presented an oral communication during the event.

Dietary probiotic supplementation improved gut amylase to trypsin ratio in European seabass reared at different rearing temperatures and survival after handling stress

PEREIRA LF, GONÇALVES J, DOMINGUES AF, PEIXOTO MD, BRAGA SF, SAKSUWAN K, SANTOS G, OZÓRIO R

Peniche, the 11th of July 2014

On the behalf of the organisation,

CAMPUS DO MAR

IPL

IPL